THE BACTERIOLOGY OF ICE CREAM WITH SPECIAL REFERENCE TO PROBLEMS OF PUBLIC HEALTH CONTROL,

being

a Thesis presented for the Degree
of Doctor of Philosophy

of

The University of Edinburgh

by

JOYCE D. CRANFIELD, B.Sc.

1952.

C O N T E N T S.

Section I. Sub-section 1. has been published in part by the author, as follows:

Cranfield J.D. (1948) The Bacteriological Examination of Ice Cream. Dairy Industries 13, 698.
ibid., 13, 800.

GENERAL INTRODUCTION.

Including: Reasons for Bacteriological Examination of Ice Cream Samples; Recorded Outbreaks of Disease attributed to Ice Cream; Methods of Manufacture; Regulations for the control of Ice Cream in America, Denmark, Sweden and Switzerland; Present-day Regulations governing the Manufacture and Sale of Ice Cream in Great Britain.

The micro-organisms which constitute the flora of ice cream are very variable in type and in number. Like the bacteria of milk, they are usually harmless, but conditions of manufacture which permit the inclusion and multiplication of large numbers of organisms in the product are the most likely to allow, at some time, contamination by pathogenic bacteria. Owing to the nutritious character of ice cream which provides an excellent medium for the growth of bacteria, these pathogenic contaminants may multiply and they or the toxic products of their metabolism may cause outbreaks of disease. For this reason the control by public health authorities of ice cream production, forms an important branch of modern hygiene.

To assist the authorities in carrying out their supervisory work, samples of ice cream are taken from time to time and tested bacteriologically, the bacterial content acting as an indication of the standard of

hygiene under which the product has been manufactured, stored and offered for sale. High bacterial counts in the final product may result from inadequate heat-treatment of the mixed ingredients and from contamination after heat-treatment by the use of unsterile equipment, from improper handling or from failure to protect the product from dust and dirt. Slow cooling, after heating or the maintenance of the "mix" at temperatures favourable to the growth of bacteria and the addition of contaminated flavouring or colouring substances, fruit juices, nuts, etc. to the product after heat-treatment may also contribute to the production of a high bacterial content in the ice cream.

Recorded Outbreaks of Disease Attributed to Ice Cream.

In view of the fact that for many years there was no adequate control of the manufacture of ice cream by public health authorities, it is not surprising that medical and public health literature have reported from time to time outbreaks of disease attributed to the consumption of contaminated ice cream.

During the last two decades of the 19th century much concern was expressed regarding the highly unsatisfactory conditions under which ice cream was being manufactured and sold to the public. The preparation

usually took place in dwelling house kitchens without adequate equipment, with no proper storage facilities and often in conditions of extreme overcrowding and dirt. Numerous cases of illness were reported which had apparently resulted from the consumption of unwholesome ice cream. During the years 1884 to 1894, the Lancet reported nine cases of this nature (ref. Lancet, 1894).

Neild-Cook (1896) refers to an enteric fever outbreak in Paisley, traced to ice cream, manufactured in a dwelling house where there was a patient suffering from typhoid fever.

Buchan (1910) quotes eight outbreaks of disease due to ice cream during the years 1875 to 1909; typhoid was reported four times, B. enteritidis, Gaertner infection twice, scarlet fever once, diorrhoea once and "ice cream poisoning" once.

Anderson (1935) reports that in America from 1908-1927 there were 36 outbreaks of disease attributed to the consumption of ice cream. They included 128 cases of scarlet fever and 6 of typhoid. He also reports that an outbreak of paratyphoid B. occurred in Aberdeen in 1925 and resulted in 23 cases of the disease. The causative organism was traced to ice cream

contaminated by a carrier. In Worcester in 1930, 24 cases of Flexner dysentery with 1 death was thought to be due to contaminated ice cream.

Savage (1938) describes a mild Sonne dysentery outbreak in 1937 in the Counties of Somerset and Dorset, in which ice cream produced under highly unsatisfactory conditions was implicated. A total of 130 cases resulted from the incriminated ice cream which was sold from a motor car over a fairly wide area.

Fuchs (1941) states that from 1934-1938, the following outbreaks were reported to the American Public Health Service:- Typhoid, 2; septic sore throat, 1; gastro-enteritis, 4; and food-poisoning,3. A total of 252 cases were involved.

Williams, Swift, Vollim and Wilson (1946) describe three outbreaks of staphylococcal food-poisoning resulting from staphylococci from the nose and hands of a cook engaged in the preparation of ice cream. Delay in cooling the heated mix and storage in a warm kitchen overnight apparently provided conditions which were favourable for the growth and toxin production of these organisms.

Finally Evans (1947) gives an account of the typhoid outbreak which occurred in Aberystwyth during

the summer of 1946. This explosive outbreak resulted from ice cream which had been contaminated by an active urinary excretor of typhoid bacilli. It involved 105 cases 4 of which were fatal.

The examples mentioned above, serve to illustrate the danger of ice cream as a vehicle of disease and the necessity for strict control of the methods of its manufacture and the conditions under which it is stored and offered for sale.

By maintaining a high standard of cleanliness during production and storage, greater safety is assured but ice cream produced under the cleanest conditions may be rendered unsafe if handled by a carrier of pathogenic bacteria. Since there is no rapid test suitable for routine purposes for determining whether ice cream is free from disease organisms, Wilson (1948) suggests that the only reliable means of avoiding the risk of contamination of this nature would be to have stricter supervision of known typhoid and paratyphoid carriers and by the routine examination for evidence of enteric infection of all personell engaged in ice cream manufacture.

Methods of Manufacture of Ice Cream.

Historical: Prior to the last war, ice cream was properly described as a dairy product, its main

constituent being milk which was thickened by heating with cornflour and sugar to form a custard which was then frozen. The other ingredients varied according to the quality of the product and sometimes included eggs, cream, flavouring and colouring substances.

One of the first reports on methods and conditions of ice cream manufacture was prepared by a special commission of the Lancet (1879) set up to examine and report on the conditions under which "penny ices" were being manufactured in the Italian quarter in London. From the description of the appalling sanitary conditions of the houses and yards in which the ice cream was being prepared, it is little wonder that outbreaks of disease, undoubtedly resulting from it, were common.

In spite of what was revealed in the report and the appeal for immediate action by public health authorities, nothing had apparently been done to improve the situation,when a second report was issued by the Lancet (1894) fifteen years later. Neild-Cook (1896) described similar conditions in the Italian quarter where cheap custard ices were manufactured and his results of bacteriological examinations of samples of the product gave plate counts of 5,000,000 organisms

per c.c. Samples of better quality ice cream prepared under more hygienic conditions in the West End of London were found to have even higher counts (8,000,000 organisms per c.c.),probably owing to the fact that whole cream was used instead of custard and was not subjected to heat-treatment. At that time heat was applied merely as a means of preparing the ice cream "mix,"not for the purpose of destroying contaminating organisms.

Wilkinson (1899) in a paper dealing with the manufacture and bacteriology of ice cream described similar conditions of manufacture and his bacteriological examinations again revealed that ice cream prepared with real cream had a much higher count than the custard ices, although the latter were being manufactured under conditions of hygiene which were far less satisfactory. The high count of the custard ice resulted from a high degree of contamination after heat-treatment,from dirty utensils and unsatisfactory surroundings whereas the high count of the cream ice was a result of the high bacterial content of the unheated cream which was the sole ingredient.

Buchan (1910) carried out an extensive investigation into the methods of preparation of ice cream

by 50 different vendors in Birmingham. He found that the custard mix was prepared in one of three ways: viz.:- (1) the milk and sugar were boiled together and then poured over the remaining ingredients in a galvanised bucket; (2) all the ingredients were heated together in a container placed in a water bath (usually a domestic boiler) and brought slowly to the boil; (3) the milk and sugar were boiled and the remaining ingredients added gradually with stirring while the temperature was maintained at boiling point. The duration of heating varied from a few minutes to over an hour. After its preparation, the custard was set out in galvanised iron buckets to cool with as large a surface as possible exposed to the air. The average duration of cooling was over 20 hours. After cooling, the mixture was strained into a freezer which was either an uncovered type stirred by hand or less commonly, a covered type with a mechanical stirrer. The freezing mixture in both cases consisted of ice and salt. Contamination occurred, in the case of the hand-stirred freezer, from the workers' hands sliding up and down the wooden handle of the stirrer. The addition of small pieces of ice to the mix to hasten freezing was another way in which contamination was brought about.

Buchan found that generally, the conditions of manufacture at that time were far from satisfactory. No separate premises were reserved for the purpose, there was no proper equipment and storage facilities were primitive or non-existent. Since the heating process, although sometimes inadequate would destroy the majority of the organisms present in the ingredients, contamination occurred mainly after heating from unsterile utensils and from exposure to dust and dirt during the cooling process, which being prolonged encouraged the multiplication of the contaminating organisms. Buchan's recommendation for a scientific method of manufacture of ice cream are interesting in view of their similarity to present day regulations which only came into force in 1947. They were for :-

1. Adequate cleansing of all vessels used for ice cream, immediately after use and proper storage facilities.

2. Manufacturers' hands, forearms and clothing to be thoroughly cleaned and kept clean throughout the process.

3. The ingredients to be boiled directly over a fire for ten minutes, or heated by means of a "water bath" at boiling point for thirty minutes.

4. The mixture to be frozen immediately after boiling, preferably in a closed freezer and thereafter to be kept frozen while in the vendor's possession.

5. No ice cream to be sold more than 48 hours after boiling.

6. The premises used for ice cream manufacture to be approved and registered by local authorities and to be constantly supervised.

From the year 1910 until 1938 the methods of manufacture of ice cream apparently changed very little, although the sanitary conditions under which it took place did improve to a certain extent as a result of Buchan's report and recommendations.

Bardsley (1938)investigated the conditions of ice cream manufacture in the Manchester district and described the methods of preparation, storage and the standards of cleanliness which she found. The ingredients were similar to those employed at the time of Buchan's investigation twenty-eight years previously, viz., milk, sugar, cornflour and eggs. Gelatine and cream were sometimes added, the gelatine because of its colloidal nature acted as a stabiliser and gave a smooth texture to the ice cream. Ice cream powders consisting of cornflour, flavoured and coloured and sometimes

sweetening were widely used. The methods of preparation of the custard mix were also similar but some manufacturers preferred to use a "cold mix" powder which consisted of dried skimmed milk, mixed with a kind of gum and powdered gelatine. Sugar and milk were added and the mixture left in a warm room to thicken. No heating was applied in this method of preparation. The dangers of this method and also the method involving the heating of the milk and sugar only, are obvious since the lack of adequate heat-treatment meant that the bacterial content of the final product was determined by the hygienic quality of the ingredients. If these were contaminated the slow cooling period or the period of thickening in a warm room, employed for the "cold mix" method, must have provided conditions which were admirable for the multiplication of any contaminating organisms. Cooling was still prolonged being rarely less than 15 hours. Freezers were similar to those described by Buchan but the most usual one was the covered type fitted with a mechanical stirrer. Although the methods of preparation of ice cream described by Bardsley in 1938 were similar to those existing in 1910, the conditions of the premises in which the product was manufactured and stored were far more

satisfactory. All large scale manufacturers were required by the local authority to have adequately constructed premises, used only for the purpose of ice cream making and storage. Some means of sterilising the equipment had to be provided and a refrigerator fitted. A few small shops still continued to manufacture ice cream during the Summer months, but the majority preferred to buy in wrapped ice cream from wholesale manufacturers, who also provided the necessary refrigerators for storage.

In spite of these improvements, Bardsley found that many samples of ice cream failed to conform to a reasonable bacteriological standard and many were grossly polluted. Apparently contaminated ingredients especially milk used for preparing "cold mixes," the lack of properly controlled heat-treatment to destroy contaminating organisms, and delayed cooling which permitted active multiplication of the organisms, were mainly responsible for the poor bacteriological standard of the products.

Present-day Methods of Manufacture:

During the war, as a result of shortages of raw materials and the increased consumption of liquid milk due to the introduction of "priority milk" schemes, making milk rationing necessary, a ban was imposed on ice cream manufacture. The shortages of milk persisted after the end of hostilities and although manufacture of ice cream was again permitted, no milk was allowed for its preparation and manufacturers found it necessary to introduce substitutes for the milk fat and milk proteins which are the basic constituents of genuine ice cream.

Margarine with edible oils and fats are substituted for milk fat or cream; lactalbumin and whey protein preparations supply the total solids, with the addition of soya flour and hydrolised cereal starch. Eggs are no longer available for use and sodium alginate usually takes the place of gelatine as a stabiliser.

This decrease in the quality of ice cream from the point of view of its palatability, has been more than offset by the improvement in its hygienic quality. To guard against the danger of food poisoning due to ice cream, the Ice Cream (Heat-Treatment etc.) Regulations,

1947 were introduced by the Ministry of Health, the three main requirements being:-(a) that the prepared mix shall not be kept at a temperature above 45°F for more than one hour before being heated; (b) that heat-treatment shall consist of heating all the ingredients at 150°F for 30 minutes or at 160°F for 10 minutes which is sufficient to destroy pathogenic organisms,and (c) that after heat-treatment the mix shall be cooled to 45°F or below within 1½ hours and kept at that temperature until freezing is begun.

As an alternative to this method of preparation, a "cold mix" may be used. This powder consists of a mixture of previously heated ingredients and requires the addition of water only, to provide the"mix"ready for freezing. It should be frozen within one hour of reconstitution.

In order that ice cream manufacturers should be able to conform to the requirements of these regulations, it is obviously necessary for them to have suitable equipment capable of accurate temperature control for pasteurising, cooling, freezing and refrigeration. The number of small scale manufacturers has lessened and more and more of the small shops which previously prepared their own ice cream prefer now to buy supplies

from wholesale manufacturers. Ice cream factories to-day, consist of large properly constructed buildings and the process of manufacture is often completely automatic and electrically controlled, so that the ice cream is not touched by hand at any time.

Preparation of the ice cream "mix," homogenisation, pasteurisation and rapid cooling by means of brine-cooled surface or plate coolers, is followed by freezing at approximately 20°F and the product is then hardened in moulds at temperatures of about 10°F.

For small-scale manufacture 10 or 20 gallon capacity units are available which mix, cool and freeze the ingredients in one single container. "Freezing", to-day, means partial freezing and incorporation of air with vigorous agitation and rapid crystallisation to produce as small crystals as possible.

Control of Ice Cream Manufacture by Public Health Authorities:-

Careful, regular supervision by public health authorities is necessary to ensure that the ice cream regulations are complied with. To help them in determining the adequacy of the methods of manufacture and conditions of storage, samples of the ice cream are submitted from time to time for bacteriological examination.

A high bacterial content in the finished product will result from:- (a) inadequate heat-treatment or (b) contamination after heat-treatment.

An unsatisfactory bacteriological result therefore requires to be followed up by careful inspection by the authorities to determine which of these two causes has been responsible. The virtual absence of milk from the product makes the phosphatase test unsuitable for determining the efficiency of pasteurisation, but since adequate heat-treatment will destroy the majority of organisms present in the mix, the results of bacteriological examinations of samples taken before and immediately after heat-treatment, give information regarding the adequacy of the treatment. Where the second result is a satisfactory one, samples taken at various stages thereafter will determine the source of contamination producing the high bacterial content in the finished article.

The elaborate plant now generally used for ice cream manufacture requires careful cleansing and sterilisation, otherwise it may become highly potent as a source of contamination, and where ice cream is not packed mechanically but served by hand, another potential source of contamination is provided,and careful

attention must be paid to the personal cleanliness of all who are engaged in ice cream work especially at this stage.

Methods of Bacteriological Examination:

The problem of choosing a single test suitable for determining the bacteriological quality of ice cream is a difficult one to solve, since each test available, has disadvantages as well as advantages attached to its use, and each requires independent interpretation. Whichever one may be employed, its main purpose must be to detect contamination and to give an indication of the extent of it. With this information, the health authority may bring pressure to bear on the manufacturer, to improve the conditions of manufacture and to remove all sources of contamination. In this way the cleanliness of the product will be assured and the danger of disease resulting from its consumption considerably lessened if not altogether avoided.

In Great Britain, no official bacteriological test has been introduced for ice cream examination since no one test is thought to be sufficiently accurate for statutory use.

Before describing the methods provisionally employed in this country at the present time, and the criticisms levelled against them, by various authorities, a brief description will be given of the regulations for the control of ice cream manufacture in force in America and some European countries,and the bacteriological methods used for testing samples there.

In considering the methods of control employed in America, it must be borne in mind that ice cream continues to be a dairy product there, and its use as a food rather than merely a delicacy is much greater than is the case in this country. Its ingredients, although basically milk and cream, are much more varied, as also is the form in which it is served; nevertheless the methods of control and the problems of bacteriological testing of American "frozen desserts" are equally applicable to our synthetic form of ice cream.

American Regulations for the Control of Ice Cream Manufacture.

The control of ice cream manufacture in America is left to the Health Authorities of the individual municipalities, in many of which legislation has been introduced in order that the requirements necessary for the manufacture of a safe and wholesome product may be enforced.

California was the first State to introduce legislation for the bacteriological control of ice cream when in 1927 they laid down a minimum standard based on a plate count of not more than 150,000 organisms per gram. As a result of an intensive campaign to encourage a high degree of cleanliness and care during manufacture the quality of the product had so improved by 1937 that they were able to reduce the minimum count allowable to 75,000 per gram.

During the years subsequent to 1927, more and more of the municipalities, including states, counties and cities introduced legal control of "frozen desserts" the name given to "any clean, frozen or partially frozen combination of two or more of the following: milk or milk products, eggs or egg products, sugar, water, fruit or fruit juices, candy, nut meats or other

harmless and wholesome food products, flavours, colour, or harmless stabiliser, and deemed to include ice cream, frozen custard, ice milk, milk sherbet, ices and other similar products". (See Frozen Desserts Ordinance and Code (1940)).

In 1935, Memphis Health Department invited the Public Health Service of the U.S.A. to help them to formulate an ordinance for controlling frozen desserts. The success of the United States, Standard Milk Ordinance and Code resulted in a demand by a number of health authorities for a similar uniform legislation to aid them in controlling ice cream production and to bring about a greater degree of uniformity of standards throughout the country. The first draft of an ordinance was therefore prepared for consideration by an appointed advisory board in 1937 and after various modifications and additions, the Frozen Desserts Ordinance /and Code (1940) was finally issued. This is not itself statutory, its form and provisions being merely a pattern for the guidance of the various municipalities in drafting their own laws. Its requirements include the provision of properly constructed buildings and plant for the preparation and storage of frozen desserts, pasteurisation of the mixes to at least 155°F for at least 30 minutes, cooling thereafter

to 50°F or less, or any other equally efficient process approved by a state health authority. It requires the bacteriological testing of at least 4 samples of frozen dessert from each plant every 6 months, the methods of testing being as laid down in "Standard Methods for the Examination of Dairy Products" 7th edition (1934).

Two forms of the ordinance are provided so that individual health authorities may choose whichever is considered the more suited to the conditions of their community. One of these provides a system of grading, whereby plants which fulfil all requirements of the ordinance are designated, Grade A. Frozen desserts from a Grade A plant should give an agar plate count of no more than 50,000 organisms per ml. Grade B plants are those where the buildings and equipment are not of such a high standard as the Grade A plants, and where the bacterial standard for the produce is based on a plate count of not more than 100,000 organisms per ml. Grade C plants are those which fail to meet the requirements of Grade B. Certain communities use Grade B merely as a penalty grade for plants where conditions at any time fail to fulfil the Grade A requirements. In communities where Grade B permits are issued Grade C is used as the penalty grade and such down-graded plants

may only operate for a period of 30 days after which time, if conditions still fail to reach a higher standard, the producer's permit may be revoked.

Some municipalities on the other hand prefer the non-grading system of ordinance whereby minimum requirements are laid down for all plants, these requirements and the bacteriological standard of the product being similar to what is required of Grade A plants.

Bacteriological examination of frozen desserts:-

Methods for testing frozen desserts are described in "Standard Methods for the examination of Dairy Products" 9th edition (1948). This work was first compiled by a committee of the American Public Health Association in 1905, to standardise methods for the bacteriological examination of milk. Nine editions have appeared since then, the later ones including sections on methods for examining dairy products and frozen desserts. For the latter, the following tests are recommended:- (1) Agar plate method; (2) direct microscopic method; (3) enumeration of coliform bacilli; (4) method of examination for haemolytic Staphylococci and beta haemolytic Streptococci. It is recommended that new supplies of frozen desserts should be tested by all the tests available and that not less

than 4 samples, each taken on a separate day from different batches of the mix, should pass the tests before a permit is granted.

The following recommendations as to the procedure to be employed in testing the samples are worthy of note:-

1. Samples.

Samples to be maintained if possible in a frozen condition from the time of sampling until testing is commenced.

2. Agar Plate Count.

Measurement of samples: In making the dilutions for the agar plate count a gravimetric method of measurement is recommended, in order to avoid the errors of the volumetric method, due to over-run, differences in densities of mixes and varying air content. The gravimetric method necessitates weighing out the product directly in the first dilution bottle containing 90 ml. sterile water, or using a weighed, sterile weighing boat similar to that used for butter analysis.

Fay (1930) on the other hand, considers that in view of the large error involved in the plate count test, any advantage gained by using a gravimetric method of measuring the sample is negligible and it makes little

difference whether 10 gm., 10 ml., or 1 ml. quantities of the samples are used.

Media:

The medium recommended for the agar plate count is Tryptone-Glucose-Beef extract Agar having the following composition:-

Agar	15 gm.
Beef extract	3 gm.
Tryptone	5 gm.
Glucose	1 gm.
Distilled water	1,000 ml.
Reaction range	pH 6.6 - 7.

This medium with the addition of 1% skim milk is the medium approved for milk examination by the American Public Health Association in 1939. Owing to insufficient data being available at the time to determine the effect of this medium on the bacteriological standards for frozen desserts, the 1940 Frozen Desserts Ordinance recommended the continued use of the medium previously used for milk testing and described in the 7th edition of "Standard Methods for the Examination of Dairy Products" (1934). This standard medium has the following composition:-

Agar	15 gm.
Beef extract	3 gm.
Peptone	5 gm.
Distilled water	1,000 ml.

The addition of glucose to a nutrient agar medium has been shown to be of advantage in producing better agreement between the plate counts calculated from different dilutions and also in producing an increase in the size of the colonies. (Fay (1926) and (1934)). The superiority of tryptone-glucose agar over standard nutrient agar was reported by Foltz and Martin (1938) and White (1938). Nelson (1943) shows how the apparent survival of heat-treated bacteria can be varied by the use of different media, such survivors of heat-treatment being apparently more demanding in their growth requirements.

Incubation:- It is recommended in "Standard Methods for the Examination of Dairy Products" (1948) that for frozen desserts agar plates should be incubated at 32°C or 35°C for 48 hours ± 3 hours.

Foltz and Martin (1938) showed that with tryptone-glucose agar there was less variation between duplicate plates incubated at 32°C than at 37°C and the counts at 32°C were significantly higher (see also

Pederson and Yale (1934); Yale and Pederson (1936); Pederson and Breed (1940)).

The Frozen Desserts Ordinance and Code (1940) recommending the continued use of the methods for bacteriological examination laid down in the 7th edition of Standard Methods, base their bacteriological standards for grading purposes on an agar plate count incubated at 37°C for 48 hours.

If it is thought that numbers of organisms are present which are incapable of growing at 37°C, additional plates should be incubated at other temperatures viz. 5°-10°C for 10 - 14 days for "psychrophylic "organisms, 18° - 25°C for 3 - 5 days for low temperature saprophytic organisms, 45°C and 55°C for high temperature and true thermophilic organisms.

Recording the plate count:

Since the plate count is merely an estimate of the number of organisms in 1 gm. which will grow under the restricted conditions of the test, it is advised that the result should be recorded as the "Standard Plate Count per gm.," the temperature of incubation being indicated.

For grading purposes the Frozen Desserts Ordinance uses the logarithmic average of the last four consecutive

counts. This avoids a false impression being created by one very high count out of four otherwise good ones, as might be the case if an arithmetic mean were used.

3. The Direct Microscopic Count.

The direct microscopic count (see Prescott and Breed (1910) and Breed (1911)) is recommended by the American Public Health Association as an additional bacteriological test for frozen desserts. It provides a check for the agar plate count and gives an indication as to the types of organisms present including yeasts and moulds and will show up contamination of organisms such as thermophiles, which may not grow under the conditions of the plate count test. The direct count may be reported as the number of clumps of bacteria present or alternatively the number of individual colonies present.

In preparing the film for the count, 0.01 ml. is withdrawn by means of a capillary pipette, with care to avoid air bubbles and spread evenly over a sq. cm. on a slide which is placed over a measured guide plate. Particles of chocolate, nuts or other particulate ingredients must be distributed evenly over the area. Very thick samples should be diluted with equal volumes of sterile skim milk before the film is made.

Alternatively a calibrated loop of 4 m.m. diameter capable of delivering 0.01 ml. of the sample may be used. After spreading, the film is dried at 40°C - 45°C on a level surface protected from dust and the fat is then removed by immersing the slide in xylol for 1 minute. After draining until dry, it is placed in 90 - 95% alcohol for at least 1 minute and again drained and allowed to dry. Staining is done by immersing the slide in carbol-methylene blue for approximately 10 - 15 seconds. By adjusting the microscope to give a field diameter of 0.206 m.m. the field area is covered by 1/300,000 ml. of milk. The bacteria (or clumps) in at least 30 fields should be counted and the total per 1 ml. calculated.

Fabian (1925) recommends a modification of the direct microscopic count for the examination of frozen desserts. His method is to weigh a slide and when it is counterbalanced, to add 0.01 grams of ice cream to either end, using a platinum loop. The ice cream is then spread evenly over an area of 1 sq. cm. and allowed to dry. He allows at least five minutes in xylol to remove the fat which is present in fairly large amounts in ice cream, and then immersion in alcohol for 20 minutes. After draining and drying on a warm surface the

film is stained with Loeffler's methylene blue for 30 seconds, and partially discolourised by dipping once or twice into 70 to 95% alcohol.

Fay (1933) considered that Fabian's gravimetric method was not sufficiently accurate. He proposed instead, pipetting 0.1 ml. of the sample on to a clean slide, adding two to four drops of sterile water and spreading evenly over the whole slide, the film then being dried and stained. By adjusting the diameter of the field to 0.157 m.m., the bacterial count may be determined by multiplying the average number of organisms per field by 1,000,000.

4. Reduction Test.

As an alternative to the agar plate count or direct microscopic count, for platform control of milk or milk products used as ingredients for ice cream, the Frozen Desserts Ordinance and Code recommend the use of the methylene blue reduction test. The method is described in Standard Methods for the Examination of Dairy Products.

For milk used for making frozen desserts in Grade A plants, the arithmetic average reduction time of four consecutive samples should be not less than 6 hours, while for Grade B plants it should be not less than 3½ hours.

For the bacteriological examination of frozen desserts or the pasteurised mix, for grading purposes, The Frozen Desserts Ordinance and Code (May 1940 edition) uses the bacterial plate count only, but for examining milk or milk products used as ingredients of the mix, either the plate count, the direct microscopic count or the reductase test may be used.
The following bacteriological standards are laid down for frozen desserts, pasteurised "mixes" and milk and milk products used as ingredients.

	Plate count. logarithmic average not to exceed (per gm.)	Direct Microscopic Count. logarithmic average not to exceed (per c.c.)		Reduction time. arithmetic average to be not less than (hrs.)
		clumps	orgs.	
Grade A plants.				
Frozen desserts and Pasteurised "mix"	50,000	-	-	
Milk or Milk products as ingredients — Raw	200,000	200,000	800,000	6
Milk or Milk products as ingredients — Heated	50,000			
Cream as ingredient — Raw	400,000	400,000	1,600,000	
Cream as ingredient — Heated	100,000			

	Plate count. logarithmic average not to exceed (per gm.)	Direct Microscopic Count. logarithmic average not to exceed (per c.c.)		Reduction time. arithmetic average to be not less than (hrs.)
		clumps	orgs.	
Grade B. plants.				
Frozen desserts and Pasteurised "mix"	100,000	-	-	
Milk or milk products Raw as ingredients Heated	1,000,000 250,000	1,000,000	4,000,000	3½
Cream Raw as ingredient Heated.	2,000,000	2,000,000	8,000,000	

5. Test for Coliform Bacilli.

No mention of the test for coliform bacilli is made in the Frozen Desserts Ordinance and Code but in the American Public Health Association's Standard Methods for the Examination of Dairy Products, methods for carrying out the test are given and it is stated to be of value, if interpreted cautiously, in checking the pasteurisation process, and in detecting contamination

subsequent to pasteurisation.

Since certain ingredients added to the mix after pasteurisation, e.g. colouring, flavouring, fruit and nuts may be highly contaminated with organisms of this group it is recommended that the test be carried out also on samples of these ingredients. It is stated that no standards can be laid down for this test to which frozen desserts should conform. Fournelle and Macy (1942) tested 69 samples of factory packed ice cream and 30 scoop samples. From their results they concluded that properly manufactured ice cream should contain less than 10 coliform organisms per ml. i.e. they should be absent from 0.1 ml.

The significance of the coliform test as a means of determining the hygienic quality of frozen desserts is debatable. Provided a freshly pasteurised mix is shown to be free of these organisms, their presence subsequently in the frozen dessert is an indication of contamination. It has been shown however that organisms may resist temperatures of pasteurisation which would prove lethal to them in milk, as a result of the protective action of sugar present in ice cream. (see Beavens (1930); Fay(1934); Fabian and Coulter(1930)). This being so their presence as an index of faulty

pasteurisation must be interpreted with caution. Fabian and Coulter found that in testing a large number of strains of coliform bacilli in ice cream, it was necessary to heat the mix to 155°F for 30 minutes to kill certain strains of Escherichia coli. According to the American Journal of Public Health, Year Book (1936-37) at that time, some cities and one state were testing for the presence of Escherichia coli in ice cream and manufacturers were finding that, in order to produce coli-free ice cream, they had to pasteurise at 160°F for 30 minutes.

6. Tests for Haemolytic Staphylococci and Beta-Haemolytic Streptococci.

Since there have been cases of food poisoning attributed to the presence of Staphylococci and beta-haemolytic Streptococci in ice cream, it is recommended that samples should be examined for the presence of these organisms by inocluating blood agar plates or slopes with loopfuls of the samples, incubating at 35 - 37°C for 48 hours and examining for and identifying any colonies surrounded by zones of haemolysis.

Summary:

The main points brought out by the study of American methods for the bacteriological control of

frozen desserts, including ice cream, and by the review of American literature dealing with the subject are as follows:-

1. It is considered desirable that samples of frozen desserts should be kept in a frozen condition until testing is commenced.

2. The agar plate count is the test most favoured by American Public Health Authorities and is the one on which are based the standards recommended by/the Frozen Desserts Ordinance and Code of the American Public Health Service.

3. A gravimetric method of measuring the sample is considered to be more accurate than a volumetric one.

4. The American Public Health Association in their 1939 edition of "Standard Methods for the Examination of Dairy Products" introduced a new agar medium prepared with tryptone and meat extract and including glucose. This medium supercedes the standard nutrient agar previously recommended.

It has been shown that the presence of glucose in a medium is of advantage in helping organisms which have survived heat-treatment to regain their vitality more rapidly. The new medium gives counts, which bear a closer relationship to the true bacterial content of

the sample and the colonies tend to belarger and more easily distinguished.

5. An incubation temperature of 32°C for 48 hours has been introduced instead of 37°C. At this temperature there has been less variation between the counts arising from different dilutions and the counts have tended to be significantly higher.

6. The direct microscopic count is recommended as a check on the agar plate count, since organisms, which may not grow under the conditions of the latter test, e.g. thermophiles, will be included in the direct count. It is also of use in identifying to a certain extent, the types of organisms present.

7. The test for coliform bacilli is considered to be of doubtful value. It has been shown that many of these organisms may resist pasteurisation temperatures in ice cream mixes as a result of the protective action of the sugar content. The presence of these organisms therefore, in freshly pasteurised mixes may not invariably indicate faulty pasteurisation. The test may be of use, however, in indicating contamination after pasteurisation in such cases where freshly pasteurised"mixes" have been found to be free of the organisms, which however appear in the product at a later stage.

8. The methylene blue reduction test is recommended only as a means of testing milk and milk products to be used as ingredients of frozen desserts, as an alternative to the plate count and direct microscopic count tests.

9. Since outbreaks of food poisoning have been attributed to Staphylococci and Streptococci in ice cream it is recommended that tests for these organisms in ice cream be included in the complete examination.

Bacteriological Control of Ice Cream in Denmark, Sweden and Switzerland.

Personal communications from Dr G. Mortensen, Veterinary Officer, Board of Health, Copenhagen, Denmark; Dr Ernst Abramson, National Institute of Public Health, Tomteboda, Sweden and Dr W. Ritter, Federal Office of Dairy Industry and Bacteriology, Liebefeld-Bern, Switzerland, gave the following information regarding methods of control of ice cream in Denmark, Sweden and Switzerland :-

1. In Denmark; Bacteriological standards to which ice cream must conform were laid down by the Ministry of Agriculture in 1951. According to these regulations, ice cream must not contain more than 100,000 organisms per ml. and coliform bacilli must be absent from 0.1 ml. Before freezing, the plate count of the mix should not exceed 30,000 per ml.

No standard technique is specified for the bacteriological examination of ice cream but the methods are fairly uniform over the whole country. In the laboratory of the Board of Health in Copenhagen, plate counts are made on nutrient agar incubated for 48 hours at 30°C. The agar contains glucose and has the following composition :-

Agar	15 gm.
Peptone	15 gm.
Glucose	2 gm.
Nacl.	5 gm.
K_2HPO_4	2 gm.
$MgSO_4$	1 gm.
Fresh whole milk	50 ml.
Water	1,000 ml.
Reaction	pH6.8.

Coliform bacilli are estimated in bile salt - lactose-peptone water containing gentian violet as an indicator of acid change. Inoculations are made with 1/10 ml., 1/100 ml. and 1/1,000 ml. quantities of ice cream, three tubes for each dilution being used.

Examination for specific pathogenic organisms is only made in cases where ice cream is suspected of being responsible for outbreaks of disease.

2. In Sweden, new government regulations controlling the sale of foods, stipulate that ice cream must be subjected to heat-treatment before freezing and afterwards stored at a temperature of not more than 4°C until freezing is commenced. The sale of ice cream refrozen after melting is prohibited.

There is as yet, no government ruling on bacteriological technique for routine examination of ice cream samples and there are no legal standards to which they should conform, but the National Institute of Public Health in Tomteboda, apply the following unofficial tests and standards:-

1. Plate count on nutrient agar after incubation for 24 hours at 37°C.

2. Plate count on nutrient gelatine after incubation at 20°C for 48 hours.

3. Estimation of the number of coliform bacilli on violet red-bile agar and in bile-salt-lactose-peptone water with crystal violet as an indicator at 37°C and 45°C the species being determined.

4. Estimation of the number of haemolytic bacteria on blood agar at 37°C.

The composition of nutrient agar was not specified.

The interpretation of the results of the above tests is as follows :

Plate counts of nutrient gelatine and nutrient agar :-

Less than 100,000 per ml. - satisfactory.

100,000 - 500,000 " " - not quite satisfactory.

More than 500,000 " " - Unsatisfactory.

Coliform bacilli at 37°C :-

Less than 100 per ml. (i.e. absent from 0.01 ml.) - satisfactory.

100 per ml. and over (i.e. present in 0.01 ml.) - unsatisfactory.

Coliform bacilli at 45°C (i.e. Typical B.coli) :-

Less than 10 per ml. (i.e. absent from 0.1 ml.) - satisfactory.

10 per ml. and over (i.e. present in 0.1 ml.) - unsatisfactory.

Examination of blood agar plates should reveal no pathogenic organisms.

3. In Switzerland there are no legal bacteriological standards for ice cream. Food regulations merely prescribe that ice cream shall be made from pasteurised and homogenized cream of a definite milk fat content. Bacteriological testing if required, is carried out by the same methods as are used in that country for the bacteriological examination of milk and other dairy products.

Regulations Governing the Manufacture and Sale of Ice Cream in Great Britain.

1. England.

Regulations for controlling the manufacture and sale of ice cream were first issued in 1947 by the Ministry of Health under the Food and Drugs Act, 1938, and together with subsequent amendment regulations they were designated the Ice Cream (Heat Treatment, etc.) Regulations 1947 to 1951. These regulations gave local authorities power to enforce certain measures of control over the methods of manufacture and storage of ice cream.

The main requirement of the regulations is that ice cream mixtures shall be submitted to adequate heat treatment. This means either that the mixture shall be heated at 150°F. for 30 minutes or, alternatively, at 160°F. for 10 minutes. After heat treatment, the mixture must be rapidly cooled to not more than 45°F. within 1½ hours and maintained at that temperature until freezing is begun, and afterwards stored at a temperature of not more than 28°F. until the time of sale. Ice cream prepared by adding water only to a complete "cold mix" is exempt from the heat-treatment requirement. By "cold mix" is meant a manufactured

product prepared by evaporating a liquid mixture which has already been submitted to adequate heat-treatment and which is sent out to ice cream manufacturers in air-tight containers.

The regulations also require that ice cream shall be protected from all forms of contamination and that all utensils and apparatus used in its manufacture, storage and distribution shall be kept in a state of cleanliness at all times.

No bacteriological standards have as yet been adopted to which samples of ice cream should conform if properly prepared in the manner laid down in the regulations. The Ministry of Health's Circular No. 69/47, which deals with the regulations, states that the Minister of Health "is still not satisfied that there is any test, the reliability of which is sufficiently established to justify its use as a statutory test, non-compliance with which would constitute an offence". But at the same time attention is drawn to a modified form of the Methylene Blue Test which is described in the Monthly Bulletin of the Ministry of Health, March 1947, and which is considered to be suitable for testing the bacterial cleanliness of ice cream. On the basis of the results of this test,

four provisional grades are suggested and where samples consistently fail to reach grades 1 or 2, it is regarded as an indication of defects of manufacture or handling and further investigation by the local authorities is advised.

2. Scotland.

In Scotland, similar regulations came into force a year later. They are known as "The Ice Cream (Scotland) Regulations, 1948", and their provisions, in addition to requiring adequate heat-treatment of the ingredients of ice cream, make compulsory the registration of premises and vehicles used in its manufacture, require satisfactory construction and condition of all ice cream apparatus and a high standard of cleanliness of the staff engaged in ice cream work. These provisions aim at preventing as far as possible contamination of ice cream or its ingredients.

The question of a statutory bacteriological test for the purpose of indicating defects of manufacture or handling of ice cream has been considered. As in the English regulations, no official bacteriological standard has been adopted, non-compliance with which would constitute an offence, since no one test is considered to be sufficiently reliable. In order to

indicate defects in the manufacture and handling of ice cream, however, it is suggested that a plate count of more than 100,000 organisms per gram or the presence of coliform bacilli in 1/100th part of a gram of ice cream may be taken as indicating the possibility of faults in manufacture, storage or distribution of the product.

The object of the first section of the present work is to determine to what extent these two suggested methods of bacteriological testing may be relied upon to show up defects in the manufacture and handling of ice cream. The results of the methylene blue reduction test provisionally adopted in England are compared with those of the plate count and the presumptive test for coliform bacilli in use in Scotland. Each test has been fully investigated and various modifications suggested.

SECTION I.

THE EXAMINATION OF ICE CREAM SAMPLES BY DYE-REDUCTION TESTS AND BY THE PLATE COUNT AND PRESUMPTIVE TEST FOR COLIFORM BACILLI.

INTRODUCTION:-

PREVIOUS STUDIES.

Nelson (1944), in America, investigated the possibility of using dye-reduction tests in the bacteriological examination of ice cream samples instead of the more orthodox plate count method.

By adding 0.1 ml. of a 0.05% solution of resazurin to each 10 ml. melted ice cream, he found that there was a general relationship between the results of the plate count test and the times required to reduce the dye to the pink stage. Samples reducing the dye within four hours at 37°C. were found with a few exceptions to have counts of more than 200,000 per ml. If the colour change at the end of three hours incubation was taken as the end point, then samples with high bacterial counts could usually be detected although there was a certain amount of overlapping of results. The same test based on a one hour period of incubation, apparently had little value

in separating samples with high bacterial counts from those with low ones.

If the dye methylene blue was used instead of resazurin, there was found to be a fairly general agreement between the times required to bring about reduction to the colourless state at 37°C. and the corresponding plate counts , but there were exceptions where plate counts of similar value were found to fall into several of the different classes into which the samples were divided on the basis of the reduction times.

The times required to bring about complete reduction of the methylene blue tended to be long, even for some samples with high plate counts.

An attempt was made to increase the rate of reduction by adding 0.2 ml. N/10 cystein hydrochloride to the ice cream and methylene blue mixture before incubation. This did in fact hasten the reaction but the relationship between the reduction times and the plate counts was lessened.

From the results of this investigation it was concluded that the methylene blue test was of little practical value for the routine grading of ice cream.

The Methylene Blue Reduction Test modified for use in the routine bacteriological testing of ice cream samples.

This test, which has been provisionally adopted by health authorities in England as an administrative aid in determining the hygienic quality of ice cream was first described in a report of the Public Health Laboratory Service staff of the Medical Research Council (1947). It consists of adding 2 ml. of the melted sample to 7 ml. of sterile quarter strength Ringer solution and 1 ml. of standard methylene blue solution as used for the examination of milk samples. The tubes are stoppered and placed in a water bath at 20°C. for 17 hours and then transferred to a water-bath at 37°C. and observed at half-hourly intervals until decolourisation of the dye is complete.

A "pre-incubation period" of 17 hours at a temperature of 20°C. was introduced into the test as a means of rendering latent infection more manifest. A period of holding at atmospheric or at a controlled temperature before actually testing the sample is the general practice in milk testing in order to distinguish between samples receiving different degrees of contamination at the time of production. The test in

this way becomes more discriminating. So, too, with ice cream samples this modification is intended to render the test more sensitive.

The report gives an account of a joint investigation into this test as a means of grading ice cream samples, by five laboratories in Cardiff, Exeter, Leicester, London and Norwich. The main advantages are considered to be its cheapness, simplicity and reproducibility and the fact that it can be carried out on large numbers of samples at the same time.

The investigators attempted to relate the results of the methylene blue test to methods of production of ice cream and a general agreement was found between the standard of hygiene of production and the reduction time. They also attempted to establish a correlation between the reduction times and the results of plate counts and tests for coliform bacilli on fresh samples, and the presence or absence of faecal coli organisms before and after pre-incubation for 17 hours at 20°C. There was a general inverse relationship between the times of reduction of the dye and the plate counts, but exceptions did occur where apparently high quality ice creams, as judged by the plate counts and tests for coliform bacilli, reduced methylene blue more rapidly than

would be expected. The results showed that the methylene blue test afforded a good indication of the presence of faecal coli in the product. Four grades were defined on the basis of the methylene blue reduction times. These are as follows :-

Grade		Hours
1.	Time taken to reduce methylene blue	4½ or more
2.	" " " "	2½ to 4
3.	" " " "	½ to 2
4.	" " " "	0 (i.e. reduction at the end of the pre-incubation period).

Laws (1948) reported on the examinations of 500 samples of ice cream by means of the modified methylene blue reduction test, the plate count test, the coliform reduction and the test for Escherichia coli. He considers that the coliform reaction is of little value as a criterion of purity, since (a) the positive reaction may be due to a mixture of organisms not of the coliform group, and (b) some coliform organisms are heat-resistant so that their presence may not necessarily indicate faulty methods. He suggests that the following standards should be applied to ice cream:-

"The numbers of colonies per ml. developing on agar at 37°C. in 48 hours shouldnot exceed 200,000.

Escherichia coli should be absent from 1.0 ml. (or for a lower standard from 0.1 ml.)"

By comparing the grades resulting from the methylene blue reduction test with the results of the plate count and Escherichia coli test based on the suggested standards, he found considerable correlation between the two sets of results in 70% of samples, in 15% there was possible correlation and in 15% no apparent correlation. In the latter cases the methylene blue test results suggested that the standard was far below that indicated by the other tests. In only a few cases did the methylene blue test suggest a standard higher than that indicated by the other tests. There were many cases in which samples showing a high degree of purity as judged by the plate count and Escherichia coli tests fell into Grade 2. He considers that certain factors in ice cream may play a part in hastening the reduction of methylene blue, and that the preparation of the ingredients and the quantities used may have a varying effect. For example, the fat content, quantity of sugar used, flavouring, etc., may all play a part in producing substances capable of reducing the dye. An essential feature of the test is the preliminary pre-incubation period which permits the multiplication of

organisms in the ice cream. Since different organisms grow at different rates the results of the test might depend,not only on the initial numbers, but also on the types of organisms present. The composition of the ice cream might also play a part in determining the nature and quantity of the bacterial content and samples of inferior nutritional value might be expected to have fewer bacteria than those with a high content of sugar and fat and consequently would appear to be more satisfactory from the hygienic point of view.

For these reasons he does not consider that the methylene blue test is sufficiently reliable to warrant its use at present as the standard method for testing ice cream samples.

In the same year, Clayson and Pirie (1948) isolated aerobic spore-forming organisms from ice cream which had been repasteurised in the laboratory and yet was still capable of reducing methylene blue in 2 to 3½ hours. Small numbers of these organisms were able to reduce methylene blue in sterile ice cream in a comparatively short time.

The rate of reduction seemed to depend to a certain extent on the composition of the ice cream since the presence of milk powder and fat tended to

accelerate the reaction. The authors therefore consider that the test is unreliable in its present form.

A second report issued by the Public Health Laboratory Service (1948) gave the results of a total of 1,685 samples of ice cream tested in 1947. A fairly good correlation between the results of the methylene blue test and those of the coliform content, faecal coli and plate count tests was reported. As the time taken to reduce methylene blue became shorter, the proportion of samples containing presumptive coliform and faecal coli organisms increased both before and after pre-incubation at 20°C and the mean plate count also increased with a decrease in reduction time. Low grading was again found to be associated with faulty cleaning and sterilising of equipment and by delayed cooling after heat treatment.

A case was reported however in which a sample with a plate count of 3,600 organisms per ml. reduced methylene blue in 4 hours. After heating in the laboratory at 70°C for 10 minutes the plate count fell to 1,000 per ml. and the result of the methylene blue test was again Grade 2. After pre-incubation at 20° the plate count of the original sample was 456,000 per ml. and of the heated sample 250,000 per ml. After

autoclaving at 115 lbs. for 10 minutes the sample proved to be sterile and the methylene blue was not reduced within 7 hours. This was given as an instance in which a few thermoduric organisms resulted in a fall to Grade 2, of ice cream which appeared to be satisfactory in other respects. The report stated that from the results of 56 samples of ice cream, tested before and after laboratory pasteurisation, it seemed that in 5 instances, thermoduric organisms might have been responsible in whole or in part for the reduction of the methylene blue since, the difference in the reduction times before and after heating was in two cases less than one hour and in three cases from one to two hours.

It was pointed out however that although thermoduric organisms may have little hygienic significance, if they are allowed to multiply unduly they may become responsible for outbreaks of food poisoning, since they are capable of producing toxins which can cause diarrhoea and sickness. It was considered that the organisms were responsible for the failure of only a small proportion of ice cream samples to reach Grade 1.

Wilson (1948) dealing with criticisms put forward against the use of the methylene blue test for

grading ice cream, considers that owing to the magnitude of the sampling and experimental errors of the plate count and coliform tests, discrepancies between the results of these tests and the methylene blue test are bound to occur. He has been unable to obtain any evidence that there are reducing substances of a chemical nature in dried milk powder, but since aerobic spore-bearing organisms are often present in that product, it is possible that they may survive heat-treatment of ice cream mixes which contain it and if present in sufficient numbers may bring about rapid reduction of methylene blue. These thermoduric organisms may multiply in the plant and survive the cleaning and sterilising process. Where ice cream which is apparently produced under satisfactory conditions is graded low by the methylene blue test, it is suggested that sanitary inspectors should arrange to have further samples tested by the plate count and coliform tests and also should satisfy themselves that the technique of cleansing and sterilising of plant and equipment is satisfactory. During 1946-47 there were only a few cases where aerobic spore-bearing bacilli appeared to be the cause of discrepancies between the results of the methylene blue test and the apparently hygienic

conditions of production. Usually where samples failed the test, a source of contamination of the plant or utensils was later discovered and when remedied fresh samples reached a higher grade. It is suggested that in order to avoid discrepancies,precautions should be taken to prevent the temperature of pre-incubation from rising above 20°C. All samples should be taken with great care to avoid contamination and transmitted to the laboratory in insulated containers within 2 hours. Frequent testing is advised and a manufacturer should not be condemned on the result of one sample alone, but should be judged on the results of a series of samples taken throughout the year. It is suggested that during the year 50% of the samples should reach Grade 1, 80% Grade 1 or 2, not more than 20% Grade 3, and none should fall into Grade 4.

Cranfield (1948) reports favourably on the methylene blue test. She found that 90% of samples with plate counts of more than 200,000 organisms per ml. and coliform organisms present in 0.01 ml. were graded as 3 or 4 by the test and 100% with counts of less than 200,000 organisms per ml. and coliform organisms absent from 0.01 ml. were graded 1 or 2. In no case was a sample with a high standard of

bacterial cleanliness as judged by the plate count test, unjustly condemned by the methylene blue test. Since a water-bath capable of maintaining a constant temperature of 20°C was not available at the time of this investigation, the samples were kept at atmospheric temperature for 17 hours before commencement of the test. Since the atmospheric temperature rarely reached 20°C and never exceeded it it, was thought that the high degree of correlation between the results of the three tests may have been favoured by the lower temperature of pre-incubation.

Cooper (1949) however favours the plate count and coliform tests for indicating the hygienic quality of ice cream, rather than the methylene blue test. He maintains that in the experience of testing authorities in Bristol during 1947 and 1948, there was agreement between the results of their own system of grading of ice cream, based on plate count and coliform tests, and those based on the methylene blue reduction times, in 73½% and 57% respectively for the two years, but there were discrepancies where the reduction test failed to pass samples which they would have considered as satisfactory. In his opinion the test also failed to show the general improvement in the hygienic quality

which appeared to have taken place during the second year. In certain firms where improvement in technique was known to have occurred, this was reflected in the plate count and coliform reactions, but not to nearly the same extent in the methylene blue results. He also suggests that the methylene blue test is more sensitive to changes in atmospheric temperature than the other two tests and considers that this is a further disadvantage.

The third report of the Public Health Laboratory Service (1949) was based on observations made in four laboratories during the summer of 1948. It was considered that the results confirmed the conclusions of the earlier reports of the Public Health Laboratory Service, that the methylene blue test is satisfactory for the routine grading of ice cream. In order to determine to what extent thermoduric organisms may influence the reduction time, a control was set up with each test. This consisted of diluted ice cream plus methylene blue as for the test, but the tube was heated at 70°C for 10 minutes before testing. If a difference of two hours or less in the reduction time was observed between the test sample and the heated control, it was assumed that thermoduric organisms

were present and were mainly responsible for the reduction. It was found that 30.7% of Grade 2 and 4.1% of Grade 3 samples were not appreciably improved by laboratory pasteurisation. Altogether approximately 10% of all samples appeared to be affected by thermoduric spore-bearing organisms of which the most common was Bacillus cereus. From the results of this investigation it was considered that although thermoduric organisms may tend to lower an otherwise Grade 1 sample to Grade 2, they are seldom responsible for a Grade 3 result. As regards the effect of the composition of ice cream on the rate of reduction, since ice cream mixes are now sufficiently nutritious to allow the growth of any bacteria, it is unlikely that the nature of the ice cream would affect the rate of growth of organisms and consequently the reduction of the dye.

The report of the Public Health Laboratory Service (1950) gives results to confirm again their previous conclusions that the methylene blue test is a satisfactory means for the routine grading of ice cream. The correlation of the results of the methylene blue test with those of the test for presumptive coliform bacilli was not however as good as that shown in the first report for "hot-mix" ice creams since

presumptive coliform organisms were present in 33.3% of Grade 1 and 52% of Grade 2 "hot-mix" ice creams, and in 19.4% of Grade 1 and 46% of Grade 2 "cold-mix" samples. However it was also found that for the "hot-mix" samples, Grades 1 and 2 were almost free from coliform organisms capable of producing gas at 44°C and of the 1.1% which were present, more than half were found by further tests not to be Bact. coli type 1, Wilson (typical B. coli), but irregular types and therefore were presumed to be not of intestinal origin. In Grades 3 and 4 however, the majority producing gas at 44°C proved to be Bact. coli, type 1. These results were considered to indicate the unreliability of the coli test for the routine control of ice cream. Since 90% of coliform bacilli in samples of Grades 1, 2 and 3 were not of the faecal type they could not be regarded as evidence of dangerous contamination. Except for plant comtrol the presumptive test for coliform bacilli was therefore considered to have little value, especially in view of the large unavoidable sampling error of the test.

In grading ice cream on the basis of the methylene blue reduction time it was decided that in order to avoid confusion, a change should be made in the

definition of Grade 1, viz. "any sample which fails to reduce methylene blue completely in 4 hours shall be classified as Grade 1". Further examination of the test at 4½ hours and over is thus avoided.

In a letter to the British Medical Journal, Hoather and Bullough (1951) states that they regard the methylene blue test only as a useful sorting test, not as reliable or informative as the bacteriological examination by plate count and coliform tests. In order to prevent variable results in the latter, they advocate that the sample should arrive at the laboratory in a frozen condition, in a sampling box packed with solid CO_2 or ice and be held in it until 3.30 p.m. when the test is commenced.

SUMMARY of PREVIOUS STUDIES.

In the foregoing review of the literature for and against the use of the methylene blue test for the routine grading of ice cream, the following seem to be the main points brought out :-

1. The test has the advantages of simplicity, cheapness and reproducibility. A large number of samples may be tested at the same time and the amount used for each is large enough to be representative of the bulk

of the sample. The methylene blue is probably influenced by all types of organisms that are able to grow under the conditions of the test but is unaffected by aggregation or clumping of the organisms.

2. There is a fairly general agreement between the results of the methylene blue test and the plate count, presence of absence of coliform bacilli and presence or absence of Escherichia coli. The majority of samples with high plate counts and coliform organisms present tend to be graded low by the methylene blue test and conversely samples with low plate counts and an absence of coliform organisms are usually graded as 1 or 2 by the methylene blue test.

3. Low grading is found in most cases to be due to faulty methods of production which when rectified, result in a higher graded ice cream.

4. Exceptions sometimes occur where samples with low plate counts fall into Grade 2 or 3 instead of Grade 1 as would be expected, and apparently satisfactory methods of production are not always reflected in high grading.

5. Samples containing apparently few aerobic spore-forming organisms only have reduced methylene blue in less than 4 hours.

Laboratory pasteurisation of some samples has not always resulted in a marked increase in reduction time of the methylene blue which suggests that thermoduric organisms have been mainly responsible for the reduction.

It is suggested that thermoduric organisms may result from :-

(a) The use of dried milk powder or other ingredient used in the preparation of ice cream containing these organisms.

(b) Inadequate cleaning and sterilising of equipment and plant which result in the multiplication and build-up of these organisms which become increasingly difficult to remove.

In view of the fact that if they are allowed to multiply unduly, they may become responsible for outbreaks of food poisoning, a lowered grading of a sample resulting from the presence of these organisms should not be objected to.

Criticisms of the presumptive test for coliform bacilli are :-

1. That a positive reaction may be due to a mixture of organisms not of the coliform group.

2. Since some coliform bacilli are heat-resistant their presence in ice cream samples may not necessarily indicate faulty methods.

3. Since 90% of coliform bacilli in samples of Grades 1, 2 and 3 were apparently not of intestinal origin their presence in ice cream could not be regarded as dangerous contamination.

The value of the test for ice cream plant control however is recognised.

Sub-Section 1. Preliminary Investigation into the Correlation between the Methylene Blue Test and the Plate Count and Presumptive Test for Coliform Bacilli.

INTRODUCTION:

The Ice Cream (Heat Treatment etc.) Regulations 1947, were issued by the Ministry of Health in England a year before the corresponding Scottish Ice Cream Regulations were introduced. At that time samples of ice cream were submitted from time to time to the Department of Bacteriology in Edinburgh University, by local authorities for bacteriological examination. Since there was at that time no official guidance on methods to be employed, the samples were tested by the same means as those employed for testing designated milks, i.e. by the plate count and presumptive tests for coliform bacilli, the standard expected being similar to that of milks of "T.T." and "Standard" grades.

After the publication of the report on ice cream testing in the Monthly Bulletin of the Ministry of Health in March, and Circular 69/47 in April, 1947, it was decided that for the examination of all further samples, the modified methylene blue test would be carried out in addition to the above two tests. During the months May to November, 100 ice cream samples were

tested in this way and it was then decided to tabulate the results of the three tests and to determine to what extent they were comparable with one another.

EXPERIMENTAL:-

Age of Samples on arrival at the Laboratory.

The majority of samples were taken from vendors in the Edinburgh Area, during the course of the afternoon, and were received in the laboratory between 4 and 5 p.m. on the same day. Five samples were received on the morning of the day following sampling but were contained in a well insulated box and arrived in a frozen condition.

The Methylene Blue Reduction Test.

The technique which was adopted in carrying out this test was similar to that laid down in the Appendix to the Report in the Ministry of Health's Bulletin. Normal saline was used as a diluent. The test was set up at 5 p.m. on the say of sampling. 2 ml. of the ice cream to be tested were added to a tube marked at 10 ml. containing 7 ml. saline and 1 ml. methylene blue solution. The tube was stoppered with a sterile bung and inverted once. The addition of ice cream to the 10 ml. mark in the tube was not difficult as the samples were

generally found to be sufficiently soft by 5 p.m. to allow of fairly easy pipetting provided a pipette with a wide enough bore tip was employed.

Pre-incubation.

Since the storing of milk samples at atmospheric temperature or at a controlled temperature before carrying out the methylene blue test is a help in distinguishing samples with different degrees of contamination, it is suggested in the bulletin that the same thing may be equally effective when applied to ice cream testing. A temperature of 20°C for 17 hours, is recommended as being the most suitable for this purpose.

When the testing of ice cream samples by this method was begun, no water bath or incubator which could be controlled at 20°C was available. The tubes were therefore stored instead at atmospheric shade temperature for 17 hours, before setting up the methylene blue test at 37°C. The tests continued through the Summer months but it is doubtful if at any time of testing the temperature exceeded or even reached 20°C. No central heating was employed during the Summer and during the latter part of the year, the heating system never raised the temperature of the laboratory to 20°C.

Precautions observed in carrying out the Methylene Blue Test.

1. All tests were carried out in duplicate (the times of reduction of the duplicate samples never varied from one another more than ½ hour and the resulting grades were invariably the same.)

2. Two colour controls were set up with each test as end-points in determining the time of reduction of the methylene blue. One, an ice cream colour control consisted of 8 ml. saline and 2 ml. of ice cream only; the other, a methylene blue control had saline, methylene blue and ice cream as in the test proper. Both these controls were boiled for 15 minutes to destroy the bacterial and enzymic reducing systems in the ice cream. Since any reduction of the methylene blue which followed boiling would be mainly due to chemical or physical factors present in the ice cream, the methylene blue control not only acted as a positive colour control, but as a precaution against the possibility of false results deriving from the above causes.

(In only one case was the control tube reduced at the same time as the test; the time of reduction was high and the sample fell into grade 1. No sample would therefore have been reported as failing the test as a result of factors other than bacterial bringing

about reduction of the methylene blue).

Method of Grading.

Samples were graded according to the time taken at 37°C for complete de-colourisation of the methylene blue. The grades depending on the times of reduction were the same as those provisionally adopted in the report i.e.:-

Grade.	Time taken to reduce methylene blue.
1.	4½ hours or more.
2.	2½ hours - 4 hours.
3.	½ hour - 2 hours.
4.	0 hours.

The Plate Count and Presumptive B. coli Test.

Technique:- After setting up the methylene blue test at 5 p.m. on the day of sampling, the remainder of each sample was refrigerated overnight at a temperature of 33° - 40°F. By the following morning the ice cream was sufficiently melted to allow of accurate pipetting without previous heating. Dilutions up to 1/1000 ml. were prepared, and duplicate petri dishes inoculated with 1 ml. of 1/1000 dilution and poured with yeastrel milk agar, were incubated for 48 hours at 37°C. Tubes containing 10 ml. MacConkey's bile-salt-lactose broth were inoculated in triplicate with 1 ml.

TABLE I.

Grading of Ice Cream Samples based on Methylene Blue Reduction Times compared with the results of the Plate Count and Presumptive Test for Coliform Bacilli.

100 Samples

Plate count thousands/ml.	Coliform bacilli in 0.01 c.c.	GRADES. 1	2	3	4
more than 200	present	·	··	:: :	:: :: :
overgrown by spreading organisms	present		·		·
overgrown by spreading organisms	absent			··	·
more than 200	absent	···	:: :	:: ·	·
less than 200	present	···	:: ·	··	···
less than 200	absent	:: :: :	:: :		

Each dot represents one sample.

The upper right hand box, outline in red on all sides indicates total number of samples failing all three tests.

The lower left hand box, outlined in red on all sides indicates the total number of samples passing all three tests.

of the 1/100 dilution and incubated for 48 hours at 37°C. The production of acid and gas in two out of three tubes was taken as indicating the presence of coliform organisms.

Comparison of Results.

The results of the methylene blue test are compared diagramatically with those of the plate count and presumptive test for coliform bacilli in Tables, I., II., III., and IV., each dot representing one sample.

In Table I., the grades depending on the results of the methylene blue test are plotted against the combined results of the plate count and presumptive test for coliform bacilli and it is seen that of 30 samples with plate counts of more than 200,000 organisms per ml. and coliform bacilli present in 0.01 ml., 27 (i.e. 90%) fall into Grades 3 and 4 of the methylene blue test and of 37 samples with plate counts of less than 200,000 and coliform bacilli absent from 0.01 c.c. 37 (i.e. 100%) occur in Grades 1 and 2. Accordingly if we take a maximum count of 200,000 organisms per ml. and B. coli absent from 0.01 ml. as the standards to which ice cream samples should conform if they are to be considered satisfactory, then 90% of those which failed to reach both standards were detected by the

TABLE II.

Grading of Ice Cream Samples based on Methylene Blue Reduction Times compared with Results of Presumptive Test for Coliform Bacilli.

100 Samples.

Coliform Bacilli in 0.01 c.c.	GRADES (based on reduction time)			
	1	2	3	4
present	....	....	....	....
absent	....	....	....	..

Each dot represents one sample.

The upper right hand box outlined in red on all sides indicates total number of samples failing both tests.
The lower left hand box outlined in red on all sides indicates total number of samples passing both tests.

methylene blue test and were graded as 3 and 4, and 100% of those samples which satisfied both standards were graded as 1 and 2. Thus, it is seen that in no case was a sample unfairly judged by the methylene blue test. Table II. shows the results of the methylene blue test compared with those of the presumptive test for coliform bacilli alone. 44 samples had coliform organisms present in 0.01 ml.; of these 33, (i.e. 75%) occur in grades 3 and 4. 8 out of 56 samples (i.e. 14%) having coliform organisms absent from 0.01 ml. fall into grades 3 and 4, but of these, 5 had plate counts of more than 200,000 and 3 contained spore-forming organisms which covered the surface of the plate and rendered counting impossible.

In Table III. the results of the methylene blue reduction test are plotted against the bacterial count given in thousands per ml., and varying from "10 and less" to more than 500. Table IV. summarises these results under two headings, i.e., those with total counts of over 200,000 per ml., and those with counts of under 200,000 per ml. From Table IV. it will be seen that 32 out of 46 samples (i.e. 70%) with counts of over 200,000 fall into grades 3 and 4 while 5 out of 49 (i.e. 10%) with counts of less than 200,000 occur in grades 3 and 4. All of these five contained coliform organisms in 0.01 ml.

TABLE III.

Grading of Ice Cream Samples based on Methylene Blue Reduction Times compared with Plate Counts.

Plate Count. thousands per m.l.	GRADES (based on reduction time)			
	1	2	3	4
more than 200	..	::::	::::	:::: :::: ::::
200 - 500	..	..	:...	..
100 - 200	..		.	..
50 - 100	...	....		.
10 - 50	:::. ::	:::. :::	.	
10 and less	::::: :::: :::: ::::	.		
Plates over-grown by spreading organisms		.	..	..

Each dot represents one sample.
The upper right hand box outlined in red on all sides, indicates the total number of samples failing both tests.
The lower left hand box outlined in red on all sides indicates the total number of samples passing both tests.

TABLE IV.

SUMMARY OF TABLE III.

Plate Count thousands per m.l.	GRADES (based on reduction time)			
	1	2	3	4
more than 200	....	....	....	....
less than 200	....	....	..	.. .
plates overgrown by spreading organisms.		.	..	..

Each dot represents one sample.
The upper right hand box outlined in red on all sides, indicates the total number of samples failing both tests.
The lower left hand box outline in red on all sides, indicates the total number of samples, passing both tests.

Discussion:-

From these reults it appears that the methylene blue test as recommended in the Ministry of Health's Monthly Bulletin, March 1947, but with a pre-incubation period of 17 hours at temperatures less than 20°C is a fairly reliable method of judging the bacterial purity of ice cream, where a maximum plate count of 200,000 organisms per ml. and the absence of coliform bacilli from 0.01 ml. is accepted as the standard to which samples should conform if they are to be considered satisfactory. Unlike the findings of some other investigators, no sample, conforming to that standard, was unfairly judged by the methylene blue test carried out in that way. Since the atmospheric temperature rarely reached 20°C and never exceeded it, the high degree of correlation may have been favoured by the lower temperature of incubation. On the other hand three samples with bacterial counts of more than 200,000 per ml. and coliform bacilli in 0.01 and a number which either failed the plate count or the coliform test/alone, were not detected by the methylene blue test, since they were graded either 1 or 2 and would therefore be considered satisfactory. Altogether, there were 22 (22%) discrepancies of this kind, 15 of which were grade 2.

It seems that the methylene blue test with a pre-incubation period at temperatures of less than 20°C, while detecting the majority of samples which were unsatisfactory either because of a high bacterial count, and/or because of the presence of coliform bacilli, is not quite sensitive enough to detect all unsatisfactory samples.

Summary:-

1. 100 samples of ice cream were tested by the modified methylene blue test as described in the Ministry of Health's Monthly Bulletin for March, 1947, and recommended for routine testing of ice cream samples in the Ministry of Health's Circular No. 69/47, but with a pre-incubation period of 17 hours at atmospheric shade temperature, not at 20°C as recommended in the bulletin. At no time did the temperature exceed 20°C.

2. The plate count and presumptive test for coliform bacilli were carried out on all samples after refrigeration overnight.

3. Correlation between the results of the three tests was good.

90% of samples with plate counts of more than 200,000 organisms per ml. and presumptive B. coli

present in 0.01 ml. were graded as 3 or 4 by the methylene blue test.

100% of samples with plate counts of less than 200,000 organisms per ml. and coliform organisms absent from 0.01 ml. were graded as 1 or 2 by the methylene blue reduction test.

4. The methylene blue test was efficient in detecting most samples with a high bacterial and coliform content. In no case was a sample with a high standard of bacteriological cleanliness unjustly condemned by the methylene blue test.

5. On the other hand, the methylene blue reduction test failed to detect 14 samples which had bacterial counts of more than 200,000 per ml. and 11 samples which had coliform bacilli present in 0.01 ml., including 3 which failed both these tests, so that there was a total of 22 samples which failed to show a correlation between the results.

6. By using a temperature of pre-incubation of less than 20°C., no sample was unjustifiably condemned by the methylene blue reduction test, but it appears that this modification tends to make the test less sensitive in detecting some ice cream samples with high total counts and/or coliform bacilli present in 0.01 ml.

Sub-Section 2. An Investigation into the possible rise in the Bacterial Content of Ice Cream Samples kept at Atmospheric Temperature for periods up to 24 hours, and the effect of such "pre-testing" periods on the Efficacy of the Plate Count and Presumptive Test for Coliform Bacilli, in judging the Purity of Ice Cream.

INTRODUCTION:

The Memorandum issued by the Department of Health for Scotland with Circular No. 43/1948 regarding the administration of the Ice Cream (Scotland) Regulations, 1948, contains a section dealing with the Bacteriological control of Ice Cream. This states that the primary purpose of sampling ice cream is to determine the hygienic quality of the article as delivered to the consumer. The result of the bacteriological examination should be read as indicating the conditions under which the ice cream has been manufactured and handled, and as a guide towards a determination of any sources of contamination. Further on it stated that "the samples should be maintained in a frozen condition from the time of sampling to the time of testing" and "if samples cannot be delivered within two hours, solid carbon dioxide or other refrigerant should be included in the transport box to ensure that the samples remain in a frozen condition during transport". It also stipulates that "if it is not convenient for the samples to be tested within an hour of

delivery to the laboratory they should be retained in a refrigerator for a period of not longer than eighteen hours".

These stipulations have apparently been made in order that the ice cream shouldbe tested in precisely the same condition as it would be in when delivered to the consumer, since as stated above the purpose of the tests is to indicate its hygienic quality when delivered to the consumer.

The tests recommended for this purpose and for indicating defects in the manufacture and handling of the ice cream are the plate count and presumptive test for coliform bacilli, the maximum bacterial count allowable being,100,000 organisms per gram and coliform bacilli to be absent from 1/100 gram.

The difficulties of maintaining the samples in the frozen state are obvious,especially as solid carbon dioxide is not always readily available to Local Authorities. It was therefore questioned as to whether there was any real necessity for the samples to be kept frozen until the time of testing and to what extent the bacterial count might increase, if at all during transit if the samples were transported in ordinary wooden transport boxes without the addition

of a refrigerant. This sub-section deals with an investigation which was primarily concerned with finding an answer to these questions, and it was hoped that more information would be gained as to the efficacy of the tests in giving the information required regarding the quality of the samples.

DETAILS OF TECHNIQUE: A total of 26 samples taken during the months January, February and March were treated in the following way :-

The samples were taken in duplicate and brought immediately to the Laboratory, their arrival being within an hour of sampling. One of the duplicates was placed in the refrigerator, the other was allowed to stand at Laboratory temperature for 24 hours, during which time the samples were tested four times.

Temperature at which the Samples were maintained :

The temperature of the refrigerator was set below freezing and checked at each time of testing. It was found that during the day, the temperature tended to fluctuate somewhat, depending on the amount of opening and closing of the door by other users of the refrigerator. Morning and night readings were usually below 30°F but during the day the temperature tended to rise occasionally above freezing-point for short periods

of time. The samples kept at laboratory temperature were placed in a cool part of a large centrally heated laboratory and the temperature noted over the 24 hours by the use of a maximum and minimum thermometer. The day temperature usually rose to 70°F and the night temperature fell to about 50°F. The average maximum temperature during the whole course of the investigation was 71°F and the average minimum 51°F. At no time did the temperature rise above 74° nor fall below 49°F.

Times of testing the Samples:

The plate count and B. coli tests were carried out on each samples between (1) 10 and 12 a.m.; (2) 5 and 6 p.m.; (3) 10 and 11 p.m. (21 samples) and (4) again the following morning, four times in all; the intervals between the times of the first three tests being approximately 6 hours and between the third and fourth tests, 12 hours: i.e. 24 hours after the first test. The counts on the samples maintained at refrigerator temperature acted as controls for the others as well as giving information on any possible rise in the bacterial count while the samples were in the refrigerator.

Dilutions in all cases were made up to 1/1000

TABLE V.

Comparison of the Results of the Plate Count and Presumptive Test for Coliform Bacilli on 26 Ice Cream Samples kept at (1) Laboratory Temperature and (2) in the Refrigerator from the time of arrival until the time of testing.

Tested -	Within 1 hour of arrival		After 6 hours		After 12 hours		After 24 hours		Methylene Blue Reduction Grade.
	Plate Count thousands/ml.	B.coli in 1/100 ml.	Plate Count thousands/ml.	B.coli in 1/100 ml.	Plate Count thousands/ml.	B.coli in 1/100 ml.	Plate Count thousands/ml.	B.coli in 1/100 ml.	
1.									
Laboratory	66	---	105	---	380	---	425	+--	4
Refrigerator	75	---	80	---	77	---	89	---	1
2.									
Laboratory	0.5	---	0.5	---	0.5	---	1.5	---	1
Refrigerator	0.5	---	0.5	---	Surface Contamination	---	1.5	---	1
3.									
Laboratory	12	---	16	---	23.5	---	40	+++	1
Refrigerator	22	---	22	---	21	---	15	---	1
4.									
Laboratory	2	---	2	---	1	---	1	---	1
Refrigerator	1	---	1.5	---	2.5	---	1	---	1
5.									
Laboratory	6.5	---	20	---	42	++-	500	++-	3
Refrigerator	5	---	6.5	---	1.5	---	2.5	---	1
6.									
Laboratory	8	---	12	---	15.5	---	9	---	1
Refrigerator	7	---	10	---	18	---	13	---	1
7.									
Laboratory	0.5	---	0.5	---	0.5	---	1.5	---	1
Refrigerator	0.5	---	0.5	---	1.5	---	0.5	---	1
8.									
Laboratory	1	---	1	---	2.5	---	5	---	1
Refrigerator	0.5	---	1.5	---	2.5	---	1.5	---	1
9.									
Laboratory	7	---	11	---	35	---	900	+++	4
Refrigerator	9.5	---	3.5	---	1.5	---	2.5	---	4
10 /									

TABLE V. (Continued).

Tested -	Within 1 hour of arrival Plate Count thousands/ml.	B.coli in 1/100 ml.	After 6 hours Plate Count thousands/ml.	B.coli in 1/100 ml.	After 12 hours Plate Count thousands/ml.	B.coli in 1/100 ml.	After 24 hours Plate Count thousands/ml.	B.coli in 1/100 ml.	Methylene Blue Reduction Grade.
10.									
Laboratory	14	---	18	---	42	---	700	---	4
Refrigerator	7.5	---	14	---	11.5	---	10	---	3
11.									
Laboratory	1.5	---	3.5	---	2	---	3	---	1
Refrigerator	0.5	---	0.5	---	1.5	---	1	---	1
12.									
Laboratory	0.5	---	0.5	---	5	---	195	---	1
Refrigerator	3.5	---	0.5	---	1	---	66	---	1
13.									
Laboratory	0.5	---	0.5	---	2	---	1.5	---	1
Refrigerator	0.5	---	1.5	---	0.5	---	0.5	---	1
14.									
Laboratory	0.5	---	0.5	---	3	---	65	---	1
Refrigerator	1	---	1	---	0.5	---	1.5	---	1
15.									
Laboratory	152	---	125	---	285	---	Uncountable	++-	4
Refrigerator	151	---	106	---	50	---	151	---	4
16.									
Laboratory	1	---	1	---	0.5	---	35	+--	1
Refrigerator	3	---	0.5	---	0.5	---	0.5	---	1
17.									
Laboratory	1	---	0.5	---	0.5	---	3	---	1
Refrigerator	1	---	0.5	---	0.5	---	1.5	---	1
18.									
Laboratory	49	---	105	---	265	---	Uncountable	---	3
Refrigerator	52	---	58	---	48	---	66	---	2

19 /

TABLE V. (Continued).

	Within 1 hour of arrival Plate Count thousands/ml.	B.coli in 1/100 ml.	After 6 hours Plate Count thousands m/l.	B.coli in 1/100 ml.	After 12 hours Plate Count thousands m/l.	B.coli in 1/100 ml.	After 24 hours Plate Count thousands/ml.	B.coli in 1/100 ml.	Methylene Blue Reduction Grade.
19.									
Laboratory	0.5	---	0.5	---	1.5	+--	5	+--	1.
Refrigerator	0.5	---	0.5	---	0.5	---	3	---	1.
20.									
Laboratory	1	---	2	---	1	+--	5	+++	1.
Refrigerator	0.5	---	0.5	---	3	---	1	---	1.
21.									
Laboratory	1.5	---	23.5	+++			500	+++	3.
Refrigerator	1	---	2.5	---			4.5	---	1.
22.									
Laboratory	1.5	---	1.5	---			3	---	1.
Refrigerator	6	---	1	---			1.5	---	1.
23.									
Laboratory	2.5	---	1.0	---			0.5	---	1.
Refrigerator	1.5	---	0.5	---			0.5	---	1.
24.									
Laboratory	1.5	+--	2.5	+--			5.5	+++	1.
Refrigerator	1.5	+--	0.5	---			1	---	1.
25.									
Laboratory	11	---	29	+--			500	+++	2.
Refrigerator	9	---	11	---			8.5	+++	2.
26.									
Laboratory	38	+--	300(approx.) (Pin point colonies)	---	Uncountable (Pin point colonies)	---	Uncountable (Pin point colonies)	---	-
Refrigerator	35	---	36	---	35	---	34	---	-

= less than

= more than.

millilitre by pipetting 10 millilitres into 90 millilitres of sterile water. Ten millilitres were chosen instead of 1 millilitre amounts as being more representative of the bulk of the ice cream which is by no means a homogeneous product. A fresh pipette was used for each dilution. When the samples were too stiff to allow of accurate pipetting, a small portion was removed with aseptic precautions to a sterile container and if necessary, placed in a 44°C water bath for a few minutes to melt.

The plates were incubated at 37°C and the colonies counted after 48 hours.

A Methylene Blue Test was carried out on each sample in the manner recommended by the English Ice Cream Regulations, for the purpose of obtaining additional information on the samples.

The results are tabulated for comparison with one another in Table V.

ANALYSIS OF RESULTS.

Samples maintained at Laboratory temperature during the Course of the Investigation:-

1. 25 out of 26 samples of ice cream tested at intervals during 24 hours, conformed to the required standard at the time of the first test by containing not more than 100,000 organisms per millilitre. All

samples passed the coliform test at the same time. The position was as follows:

16 samples (61%) had counts of not more than 5,000 organisms per millilitre.
19 " (73%) " " " " " " 10,000 " "
22 " (84%) " " " " " " 20,000 " "
24. " (92%) " " " " " " 50,000 " "

2. Samples were tested after 3 intervals of approximately 6, 6 and 12 hours respectively.

As regards the 16 samples with plate counts of less than 5,000 organisms per millilitre, the majority did not increase significantly during 24 hours since 15 of them showed no increase above 5,000 at the times of the second and third testing and 12 of them were practically unchanged 24 hours after arrival of the samples. Of the four samples which did show an increase in numbers, this occurred in the following way:

	1st Test	2nd Test	3rd Test	4th Test
(1)	1,500	23,500	-	500,000
(2)	500	500	5,000	195,000
(3)	500	500	3,000	65,000
(4)	1000	1000	500	35,000

Thus a marked increase is detected 24 hours after arrival of the samples. The plate counts of two of these samples increased to more than the permissible amount and the samples would therefore fail the test after 24 hours, although the original counts were 1,500 and 500 per millilitre respectively, at the time of the first testing.

3. Although there was no significant rise in the bacterial content of the majority of samples with initial counts of less than 5,000 organisms per millilitre, samples with counts above 5,000 showed a steady rise over the 24 hours in 9 out of 10 cases. Two of 3

samples with initial counts of between 5,000 and 10,000 failed at the fourth test with counts of more than 500,000 per millilitre. The third one showed no significant rise, the initial count being 8,000 and the final one 9,000 organisms per millilitre.

Two of 3 samples with initial counts of between 10,000 and 20,000 failed the fourth test with counts of more than 500,000 per millilitre. The third one had an initial count of 12,000 which rose to 40,000.

Of the 2 samples with counts of between 20,000 and 50,000 per millilitre, both failed the second test with counts of 300,000 and 105,000, which subsequently rose to be uncountable after 24 hours.

Thus of the 9 samples passing the initial test but with count of more than 5,000 per millilitre, three failed the second and third tests and seven the fourth test.

4. B. coli Test: The first test on every one of the 27 samples of ice cream failed to show B. coli in 1/100 ml. There were only slight changes in the results of the 2nd and 3rd tests, one sample proved to be positive by the 2nd test and one other by the third, but after 24 hours B. coli was detected in 8 samples. Each of these 8 samples showed a rise in the bacterial counts,

five of which were above the limit of 100,000 organisms per millilitre at the time of the 4th testing.

Thus it appears that those samples showing a rise in bacterial count over 24 hours also tend to show a rise in the B. coli content.

A high/bacterial count and the presence of B. coli may not be detected if the samples are tested immediately after refrigeration. Even testing after 6 or 12 hours standing at room temperature may fail to show them but a delay in testing of 24 hours will differentiate these samples from those in which little multiplication occurs and where B. coli continues to be absent from 1/100 ml.

5. Refrigerated Samples: No significant changes either in the plate counts or the content of coliform bacilli, resulted from holding the samples for periods of up to 24 hours at refrigerature temperature.

6. Methylene Blue Reduction Test: The Methylene Blue Reduction Test carried out in the manner recommended by the English Ice Cream Regulations allows for a pre-incubation period at 20°C of 17 hours before the actual test is carried out. The purpose of this is to distinguish between different degrees of contamination of the samples or to show up latent contamination which may have occurred after heat-treatment.

It is of interest therefore to compare the results of the methylene blue test with the results of the Plate count and Coliform tests carried out at the specified intervals of time and to note that those samples showing low bacterial counts at the first testing and no marked rise at subsequent tests invariably fell into Grade I. of the methylene blue test, whereas the 7 samples graded as 3 or 4 showed the following increases in bacterial content.

	Methylene Blue Reduction Grade.	1st Test Bact. Count.	B.coli	2nd Test Bact. Count.	B.coli	3rd Test Bact. Count.	B.coli	4th Test Bact. Count.	B.coli
(1)	4	66,000	—	105,000	—	380,000	—	425,000	
(2)	3	6,500	—	20,000	—	42,000		500,000	
(3)	3	1,500	—	23,500				500,000	
(4)	4	7,000	—	11,000	—	35,000	—	900,000	
(5)	4	14,000	—	18,000	—	42,000	—	700,000	
(6)	4	152,000	—	125,000	—	285,000	—	Uncountable	
(7)	3	49,000	—	105,000	—	265,000	—	Uncountable	

It is noticeable in four of the above that the results of the methylene blue reduction test bear little correlation with the results of the bacterial counts and B. coli tests in the first instance, but the correlation become closer at the second and third tests, and at the time of the fourth test the results are quite comparable.

DISCUSSION:

Under the ice cream regulations, except where manufactured from cold mixes, ice cream must undergo adequate heat-treatment in order to kill the majority of bacteria including any pathogenic organisms which may be present. It must also be kept free from contamination during storage and distribution.

A satisfactory bacteriological test should be able to detect faults in both the efficiency of the heat-treatment and in the methods of handling the product after manufacture.

From the results of this investigation it appears that the bacterial count and presumptive test for coliform bacilli, when carried out immediately after sampling may show whether adequate heat treatment has or has not been carried out, although it is thought that the maximum count of 100,000 organisms per millilitre which is permitted under the regulations sets too low a standard. It was found that 22 out of 26 samples i.e. 84%, had bacterial counts of not more than 20,000 per millilitre when tested shortly after they were received in the laboratory and of these 16 had counts of not more than 5,000. A sample with a count of 50,000 to 100,000 could not therefore be considered to have

undergone adequate heat treatment if compared with the above samples on the basis of these bacterial counts.

It is doubtful if the bacterial count and coliform tests carried out on samples in the frozen state can reveal contamination which occurs between heat treatment and refrigeration. If ice cream which is rendered almost sterile through adequate heat treatment becomes exposed to contamination by unsterile containers or faulty handling, the organisms will not multiply at the temperature of the frozen ice cream which is below 28°F, but will remain dormant and may not be detected in the 1/100 millilitre removed for testing. A low bacterial count does not therefore mean that the product is free from a latent contamination. It is essential from a hygienic standpoint that any sources of contamination should be revealed since they may harbour pathogenic organisms. Apparently this can only be done if the samples are allowed to stand at atmospheric temperature, or at a controlled temperature for a period to allow the contaminants to develop to such an extent that they may be shown by the bacterial count and presumptive test for coliform bacilli.

The 16 samples with counts of not more than 5,000 organisms per millilitre had apparently been taken from ice cream which had been adequately heat-treated, and the majority of them showed little change in the count even after 24 hours standing at laboratory temperature, 4 of them however, did increase during the 24 hours and 2 of these failed to reach the standard of not more than 100,000 organisms per millilitre after the 24 hours period. These 4 can not therefore be considered to be of such a high standard as the other 12 which showed little or no rise in bacterial count over the same period. They must either have contained initially a number of thermoduric organisms rendered dormant by the treatment, and only capable of multiplication after a period at atmospheric temperature or, which is more likely, especially in the case of the two showing considerable increase in numbers after 24 hours, the ice cream has been subjected to contamination after heat-treatment either through unsterile containers or through faulty handling.

The pre-testing period of the methylene blue test was designed to show up latent infection. If the plate count is to be continued to be used for the routine examination of ice cream, a similar period

of standing at a controlled temperature prior to testing might prove to be advantageous in separating samples which have received different degrees of contamination during production. In other words the test might be rendered more sensitive by such a modification and thereby be of greater value in tracing faults in production. Certainly one would expect closer agreement with the results of the methylene blue test if this were done. The argument that by so doing ~~xx~~ one would no longer obtain a correct picture of the bacterial content at the time of consumption of the ice cream is hardly justified, since it is possible that a test carried out immediately after the sample has been in the frozen state may not show up organisms which, although actually present, may be "attenuated" by the heating, cooling and freezing processes.

SUMMARY.

1. In order to determine the effect of pre-testing periods at atmospheric temperature, on the efficacy of the plate count and presumptive test for coliform bacilli, in judging the quality of ice cream samples, 26 samples were tested immediately after being in a frozen condition and 6, 18 and 24 hours after melting.

2. Twenty-five out of 26 samples passed the provisional standard, by having not more than 100,000 organisms per ml. when tested immediately after melting. Sixteen had plate counts of less than 5,000 per ml. Coliform bacilli were absent from 0.01 ml. in all cases.

3. Thirteen samples showed no rise or only a slight rise during 24 hours at atmospheric temperature. In 10 others however, the count rose to over 100,000 per ml. after 24 hours, and 8 had coliform organisms present after that time.

4. Four of the 16 samples with initial counts of not more than 5,000 per ml. showed marked increases in the plate counts during the 24 hours, and in 2 of them the counts rose above 100,000 per ml.

These samples although apparently adequately heat-treated may have been contaminated afterwards by organisms which remained dormant at freezing temperature, and which were revealed by the plate count test only after the samples had stood for a time at atmospheric temperature.

5. Samples with low bacterial counts after pre-testing periods up to 24 hours, invariably fell into Grade 1 when tested by the methylene blue reduction test.

Of 7 samples of Grades 3 and 4, 1 had a bacterical count of more than 100,000 when first tested and all had counts of over 400,000, 24 hours later. Although when first tested 4 of them showed little correlation between the results of the methylene blue test and the plate count and coliform tests, after the samples had stood for 24 hours at atmospheric temperature the results of the tests were comparable in all cases.

6. If the plate count and presumptive test for coliform bacilli are to continue to be used for testing ice cream samples, it is suggested that a period at atmospheric temperatures before testing would render the tests more sensitive in detecting contamination, and in distinguishing between samples with few organisms present and those with greater numbers of "attenuated" organisms, some of which may have gained access to the product after heat-treatment, but which remain dormant for a time as a result of freezing.

7. If it were decided to allow a pre-testing period at atmospheric temperature before testing, then the time taken for samples to arrive at the laboratory could be included in this period, in which case no refrigerant during transport would be necessary except at such times of the year when the atmospheric temperature might rise above 20°C.

Sub-section 3: Correlation between the Methylene Blue Reduction Times and the Plate Counts of Samples of Ice Cream, tested immediately after they have been in a Frozen Condition, and after being held at 20°C for 6, 12, 18 and 24 hours.

INTRODUCTION:

In considering the discrepancies reported by some workers, between the results of the plate count test and those of the methylene blue reduction test, it is considered that the following points are worthy of note:-

(a) In all cases, the plate count tests were carried out on samples, presumably tested shortly after being in a frozen condition.

(b) The methylene blue test on the other hand was carried out in the manner recommended in the 1947 Bulletin of the Ministry of Health, viz. after the samples had been held for 17 hours at a temperature of 20°C. Under these circumstances complete agreement between the results can hardly be expected.

The purpose of the pre-incubation period as applied to the methylene blue test was to show up latent infection. It has been shown in the previous sub-section that holding samples for a period before carrying out the plate count test, serves to separate out samples with different degrees of contamination.

In order to investigate further the conditions which will give the closest correlation between the results of the two methods of testing, the following series of tests were carried out on 280 samples of ice cream. These were all tested first of all by the plate count method immediately after being frozen. One hundred of them were again tested after being held at 20°C for 6 hours, 61 after 12 hours at 20°C, 68 after 18 hours at 20°C, and 104 after 24 hours at 20°C.

The correlation of these results with the grading based on the methylene blue test is shown diagrammatically in Tables VI., VII., VIII., IX. and X.

All samples tested were those sent in to the Department of Bacteriology of Edinburgh University by public health authorities in Edinburgh and the South-Eastern counties of Scotland for routine examination.

ANALYSIS OF RESULTS:

(1) Out of 280 samples, 218 had plate counts of less than 100,000 orgs./ml.

41 had plate counts of more then 100,000 orgs./ml.

21 had numerous pin point colonies present on the plates.

i.e. If 100,000 orgs./ml. is maximum count permissible

TABLE VI

Plate counts of samples of ice cream tested immediately after arrival at the laboratory in a frozen condition compared with Grades based on results of the Methylene Blue reduction Test.

280 samples.

Plate Count thousands/ml.	GRADES (based on Methylene Blue Reduction Times) 1.	2.	3.	4.
< 1 - 1	29	8	1	
1.5 - 20	28	9	3	3
21 - 50	5	3	3	3
51 - 100	1	2	4	4
101 - 200	1	3	3	4
> 200		3	3	8
0 - 50 + p.p.c.	2	4	4	1
51 - 100 + p.p.c.				1
101 - 200 + p.p.c.				3
Numerous p.p.c.	3	2		

Each dot represents a single sample. < = less than, > = more than, ∝ = numerous, p.p.c. = pin point colonies.

The upper left box, outlined in red on all sides represents total number of samples passing both tests.

The lower right box outlined in red on all sides represents total number of samples failing both tests.

for samples tested immediately after arrival in the laboratory 77.8% of the total number would pass the required standard.

(2) Out of 280 samples 192 were graded 1 or 2 according to the methylene blue test and 88 were graded 3 or 4.

i.e. 68% of the samples would pass the methylene blue test.

(3) Grade 1 included only samples (131) with counts of 100,000 orgs./ml. or less, with 1 exception (and 5 with numerous pin point colonies present on the plates.

Grade 2 included only samples (42)with counts of 100,000 orgs./ml. or less with 6 exceptions (and 7 with numerous pin point colonies present on the plates).

Grade 3 included 6 samples with counts of more than 100,000 orgs./ml. and 23 with counts of less than 100,000 orgs./ml. (and 4 with pin point colonies present on the plates.)

Grade 4 included 28 with counts of more than 100,000 orgs./ml. and 22 with counts of less than 100,000 orgs./ml.

i.e. 45 Samples with counts of less than 100,000 orgs./ml. were graded 3 or 4 by the methylene blue test.

Although practically all samples graded 1 or 2 by means of the methylene blue reduction test had correspondingly satisfactory plate counts, many of the grade 3 and 4 samples on the other hand had apparently low bacterial counts and would be considered satisfactory if judged by the results of the plate count test alone. Good correlation between the results of the two tests only occurred with samples of high grade as judged by the methylene blue results.

Altogether with 18% of the samples there was no correlation between the results of the two tests, 16% being samples graded 3 or 4 by the methylene blue test having plate counts of less than 100,000 orgs./ml. These discrepancies may be due either (a) to the ability of the methylene blue test to show up latent infection due to contamination which is undetected by plate counts prepared immediately after the sample has been in a frozen condition or (b) it may result from reduction of the dye by a few active organisms or (c) a few organisms may multiply so rapidly that they are sufficient in numbers after 17 hours at 20°C to bring about rapid reduction of the methylene blue. A close correlation can hardly be expected between tests carried out on a sample maintained under such widely different conditions of time and temperature.

TABLE VII

Plate counts of samples of ice cream held at 20°C for 6 hours after arrival at laboratory compared with grades based on the results of Methylene Blue Reduction Test.

100 samples.

Plate Count thousands/ml.	GRADES (based on Methylene Blue Reduction Times) 1.	2.	3.	4.
< 1 - 1	:::: :::: :::	::		
1.5 - 20	:::: :::: :::: :::: :::: :::: ::::	::::		.
21 - 50	::::	.	::::	.
51 - 100		::	.	.
101 - 200			::	:::
> 200		::	::::	:::: ::::
0 - 50 + p.p.c.	::	::	::	:::
∝ p.p.c.				::

Each dot represents a single sample. < = less than, > = more than,
∝ = numerous, p.p.c. = pin point colonies.
The upper left box, outlined in red on all sides represents total number of samples passing both tests.
The lower right box outlined in red on all sides represents total number of samples failing both tests.

The question is however, which test can be relied upon the more to detect contamination in the ice cream. In this case the dye test would condemn 44 samples as unsatisfactory which, from the point of view of the plate counts were apparently of a high standard. Are the reasons for this significant from a public health point of view ?

II. The results given diagrammatically in Table VII. are of 100 samples tested by the plate count method after standing for 6 hours at 20°C. These are plotted against the resulting grades of the methylene blue test to determine whether closer agreement between the results of the two tests may be obtained by allowing a "pre-testing" period at atmospheric temperature to elapse before plating.

(1) Out of 100 samples, 69 had plate counts of less than 100,000 per ml.

20 had plate counts of more than 100,000 per ml.

11 had numerous pin point colonies present on the plates.

i.e. If 100,000 orgs./ml. was the maximum count permissible for samples tested after standing for six hours at 20°C., 69% of these would pass the required standard.

(2) Out of 100 samples 67 were graded 1 or 2 according to the results of the methylene blue test and 33 were graded 3 or 4.

i.e. 67% of the samples pass the methylene blue test.

A similar total would therefore be considered satisfactory when judged by either test.

Grade 1 included only samples (49) with plate counts of 50,000 or less (and two with numerous pin point colonies present on the plates.)

Grade 2 included only samples (12) with plate counts of 100,000 or less with two exceptions having more than 200,000 orgs. per ml. (and two with pin point colonies present on the plates.)

Grade 3 included 6 samples with plate counts of more than 100,000 and 5 samples having less than 100,000 orgs. per ml. and two with pin point colonies on the plates.

Grade 4 included only samples with plate counts of more than 100,000 orgs. per ml. with three exceptions with counts less than 100,000 (and five with pin point colonies on the plates.)

Thus altogether 10% of the samples showed discrepancies between the results of the plate count and methylene blue tests.

TABLE VIII.

Plate counts of samples of ice cream held at 20°C for 12 hours after arrival at laboratory compared with grades based on the results of Methylene Blue Reduction Test. 61 samples.

Plate Count thousands/ml.	GRADES (based on Methylene Blue Reduction Times).			
	1.	2.	3.	4.
< 1 - 1	:::: :::: :::			
1.5 - 20	:::: :::: .	...		
21 - 50	:::·	·	·	··
51 - 100	...	·		·
101 - 200			·	
>200	·		...	:::·
0-50 + p.p.c.	··			
∝ p.p.c.	·			

Each dot represents one sample. < = less than. > = more than.
∝ = numerous. p.p.c. = pen point colonies.

The upper left box outlined in red on all sides represents total number of samples passing both tests.

The lower right box outlined in red on all sides represents total number of samples failing both tests.

Eight of the ten discrepancies were due to samples graded 3 or 4 by the methylene blue test having plate counts of less than 100,000 orgs./ml. abd 2 were samples with plate counts of more than 200,000 orgs./ml. graded 2 by the methylene blue test.

It is apparent that the correlation between the results is closer here than was the case with samples tested by the plate count test immediately after being frozen.

III. Although a "pre-testing" period of 12 hours at 20°C is not practicable as the timing would be too inconvenient for routine work, 61 samples were tested after this time by the plate count method and the results compared with those of the methylene blue test. These are given in Table VIII.

(1) Out of 61 samples, 46 had plate counts of less than 100,000 per ml.
12 had plate counts of more than 100,000 per ml.
3 had numerous pin point colonies present on the plates.

i.e. If 100,000 orgs. per ml. was the maximum count permissible for samples tested after 12 hours, at 20°C, 75.4% would pass the required standard.

2. Out of 61 samples 46 were graded 1 or 2 by the methylene blue test and 15 were graded 3 or 4. i.e. 75.4% passed the methylene blue test.

Grade 1 included 37 samples with plate counts of less than 100,000 orgs. per ml., 1 sample with a plate count of more than 100,000, and 3 samples with numerous pin point colonies present on the plates.

All samples in Grade 2 had plate counts of less than 100,000 per ml.

Grade 3 included 4 samples with counts of more than 100,000 and 1 with a count of less than 100,000.

Grade 4 included 7 samples with counts of more than 200,000 per ml. and 3 with counts of less than 100,000 per ml.

Thus omitting these samples which gave pin point colonies on the plates, 5 samples (8.1%) showed no correlation between the results of the two tests, 4 being graded by the methylene blue test lower than would be expected from the results of the plate count test and 1 higher.

It is seen, therefore that the results of the methylene blue test and the plate counts of samples held for 12 hours at 20°C show closer correlation with one another than was shown in either of the two

TABLE IX

Plate counts of samples of ice cream held at 20°C for 18 hours after arrival at laboratory compared with grades based on results of the Methylene Blue Reduction Test.

68 samples.

Plate Count thousands/ml.	GRADES (based on Methylene Blue Reduction Times)			
	1.	2.	3.	4.
<1 - 1	•••			
15 - 20	•••• •••• •••	•••		
21 - 50	•••• ••	••	•	
51 - 100	•••	••		
101 - 200	••	•••		
>200		•••• •••	•••• •••	•••• ••••
0 - 50 + p.p.c.	•••	•		
50 - 90 + p.p.c.				••
100 - 200 + p.p.c.				
∝ p.p.c.	•••			

Each dot represents a single sample. < = less than, > = more than, ∝ = numerous, p.p.c. = pin point colonies.

The upper left box outlined in red on all sides represents the total number of samples passing both tests.

The lower right box outlined in red on all sides represents the total number of samples failing both tests.

previous series of results. 75% of the total number of samples were passed by both tests, but the methylene blue test still seems to be slightly more severe in judging the samples than the plate count test carried out at that time.

IV. Table IX. shows the plate counts of 68 samples of ice cream tested after standing at atmospheric temperature for 18 hours compared with the grading based on the results of the methylene blue test.

(1) Out of 68 samples, 31 had plate counts of less than 100,000 per ml.

28 had plate counts of more than 100,000 per ml.

9 had numerous pin point colonies present on the plates.

i.e. If 100,000 orgs./ml. was maximum count allowable for samples tested after 18 hours at atmospheric temperature 45% could pass the required standard.

(2) Out of 68 samples 49 were graded 1 or 2 by the methylene blue test and 19 were graded 3 or 4.

i.e. 72% pass the methylene blue test.

Grade 1 included 23 samples with plate counts less than 100,000, 2 samples with plate count between

100,000 - 200,000 (and 6 samples with numerous pin point colonies present on the plates.)

Grade 2 included 7 samples with plate counts less than 100,000, 10 with plate counts over 100,000 (and 1 with pin point colonies present on the plates.)

Grade 3 included only samples (7) with counts of more than 200,000 orgs. per ml. with one exception.

Grade 4 included only samples (9) with plate counts of more than 200,000 per ml. (and two with pin point colonies present on the plates.)

Thus all samples with plate counts of less than 100,000 orgs. per ml. were graded 1 or 2 by the methylene blue test with one exception in grade 3 which had a count of between 21,000 and 50,000. With one exception all samples graded 3 or 4 had plate counts of more than 200,000.

12 Samples (17%) however had plate counts of more than 100,000 and were graded 1 or 2 by the methylene blue test.

A total of 13 (19%) show no correlation between the results of the two tests. In this case it seems that a pre-testing period of 18 hours at atmospheric temperature before plating makes the test more severe than the methylene blue test, since only 45% of the

Plate counts of samples of ice cream held at 20°C for 24 hours after arrival at laboratory compared with grades based on results of the Methylene Blue Reduction Test.

104 samples.

Plate Count thousands/ml.	GRADES (based on Methylene Blue Reduction Times) 1.	2.	3.	4.
< 1 - 1	::··			
1.5 - 20	:::: :::: :::: ::	·		
21 - 50	:···	··		
51 - 100	····	·		
101 - 200	·	·	·	
> 200	:··· ···	:···	:··· ·	:··· :··· :··· :··· ·
0 - 50 + p.p.c.	:··· ·	·		
51 - 100 + p.p.c.	··	·		
101 - 200 + p.p.c.	··		·	·
∝ p.p.c.	·			

Each dot represents a single sample. < = less than, > = more than, ∝ = numerous, p.p.c. = pin point colonies.

The upper left box outlined in red on all sides represents the total number of samples passing both tests.

The lower right box outlined on all sides in red represents the total number of samples failing both tests.

samples would pass as against a 72% by the methylene blue test.

V. Finally, a series of tests were carried out on 104 samples tested 24 hours after arrival in the laboratory during which time they were maintained at atmospheric temperature. Results are given in Table X. along with the results of the methylene blue test.

(1) Out of 104 samples 41 had plate counts of less than 100,000 per ml.

49 had plate counts of more than 100,000 per ml.

14 had numerous pin point colonies on the plates.

i.e. If 100,000 orgs/ml. was maximum count allowable for samples tested after 24 hours at atmospheric temperature 39% would pass the required standard.

(2) Out of 104 samples 67 were graded 1 or 2 according to the methylene blue reduction test and 37 were graded 3 or 4.

i.e. 64% pass the methylene blue test.

Grade 1 included 37 samples with plate count less than 100,000, 8 with plate counts of more than 100,000 (and 10 with numerous pin point colonies on the plates.)

Grade 2 included 4 samples with plate counts less than 100,000, 6 with plate counts of more than 100,000 (and 2 with numerous pin point colonies on the plates).

Grade 3 included only samples (10)with plate counts of more than 100,000 (and 1 with numerous pin point colonies present.)

Grade 4 included only samples (25) with plate counts of more than 100,000 (and 1 with numerous pin point colonies present.)

Thus all samples with plate counts of less than 100,000 orgs./ml. were graded 1 or 2 by the methylene blue test without exception.

14 Samples (13%) which failed the suggested plate count standard by having more than 100,000 orgs. per ml. were graded 1 or 2 by the methylene blue reduction test. These 14 constitute the total number of samples showing discrepancies between the results of the two tests.

It seems that the holding of samples for as long as 24 hours before carrying out the plate count test makes the test much more severe for judging the quality of ice cream samples than is the methylene blue test. Since too many samples would fail to pass the test,it is considered that it would not be satisfactory for routine

testing of ice cream. It seems that the holding of samples for as long as 24 hours before testing results in too many high plate counts and the test ceases to be discriminating. Its correlation with the methylene blue test is considerably less as a result of the long pre-testing period which cannot be recommended for routine testing of ice cream.

SUMMARY OF RESULTS OF TESTS REPRESENTED DIAGRAMMATICALLY IN TABLES VI., VII, VIII., IX. and X.

1. The plate counts of samples tested immediately after being in a frozen condition showed correlation with the grading based on the results of the methylene blue test in 82% of the cases examined. Of the 18% which failed to show any correlation between the results, the majority (86%) of the discrepancies were due to samples with plate counts of less than 100,000 orgs. per ml. being graded 3 or 4 by the methylene blue test.

2. Samples tested by the plate count method after being held for 6 hours at a temperature of 20°C and by the methylene blue test showed correlation between the results of the two tests in 90% of the total number tested. Of the 10% showing no correlation, 80% were due to samples with plate counts of less than 100,000

orgs. per ml. being graded 3 or 4 by the methylene blue test.

3. Samples tested by the plate count method after being held for 12 hours at a temperature of 20°C and by the methylene blue test showed correlation between the results of the tests in 91.8% of the total number tested. Of the 5 which showed no correlation between the results of the tests, 4 of the discrepancies were due to samples with less than 100,000 orgs. per ml. being graded 3 or 4 by the methylene blue test.

4. Samples tested by the plate count method after being held for 18 hours at 20°C and by the methylene blue test, showed correlation between the results of the tests in 81% of the total number examined. Of the 19% showing no correlation, all but one of the discrepancies were due to plate counts of more than 100,000 orgs. per ml. being graded 1 or 2 by the methylene blue test.

5. Samples tested by the plate count method after being held for 24 hours at 20°C and by the methylene blue test, showed correlation between the results of the two tests in 87% of the total number tested. All the discrepancies were due to samples with plate counts of more than 100,000 orgs. per ml. being graded 1 or 2 by the methylene blue test.

6. The closest correlation between the results of the two tests was found to occur when the plate counts were set up on samples which had been held for 12 hours at 20°C. Such a timing for the test however is obviously impracticable for routine purposes. With that exception, correlation between the results was closest when the samples were plated out after 6 hours at 20°C.

Results obtained from testing in this way show that the plate count is slightly more lenient than the methylene blue test.

By holding samples for 18 hours or 24 hours before carrying out the plate count test, too many of the samples fail and the test is therefore too exacting to be of value in routine testing. The methylene blue test on the other hand, carried out after a pre-incubation period of 17 hours passes on an average about 65% of samples. It is therefore more discriminating than the plate count test carried out immediately after the samples have been frozen and probably more sensitive in showing up contamination in the ice cream. It is also more severe than the plate count test on samples tested after 6 hours at atmospheric temperature, but the results approximate more closely with one another in this case.

Sub-section 4: Correlation between the Results of the Methylene Blue Test and those of the Presumptive Test for Coliform Bacilli, carried out on samples of Ice Cream immediately after melting and after 6, 18 and 24 hours at 20°C.

TECHNIQUE:

Two hundred and eighteen samples of ice cream were tested by the methylene blue test in the manner recommended by the English Public Health Authorities. They were also tested immediately after melting at room temperature by the presumptive test for coliform bacilli.

Eight of them were again tested for coliform bacilli after standing at atmospheric temperature of not more than 20°C for a period of 6 hours; 62 after 18 hours and 99 after 24 hours.

In carrying out the test for coliform bacilli, 1 ml. of 1 in 100 dilution of the sample was added to each of three test tubes containing 10 ml. MacConkey broth. After incubation at 37°C for 48 hours, the presence of acid and gas in two or three of the tubes was presumed to be evidence of the presence of coliform bacilli in 0.01 ml. of the sample.

RESULTS:-

The results of the methylene blue reduction test divided the 218 samples into the following grades :-

Grade 1	112 samples
Grade 2	36 "
Grade 3	24 "
Grade 4	46 "
Total	218 "

70 Samples failed to reach the standards for Grades 1 and 2 and would be considered as failing the provisional test for ice cream recommended by the Ministry of Health. This constitutes 32% of the total number of samples tested by this method.

The results of the presumptive test for coliform bacilli carried out on samples of different ages are given in the following Table:-

Samples tested	No. of Samples tested	Coliform bacilli present in 0.01 ml.	Coliform bacilli absent from 0.01 ml.
Immediately	218	43 (19.7%)	175 (80.3%)
After 6 hours	80	22 (27.5%)	58 (72.5%)
After 18 hours	62	35 (56.4%)	27 (43.5%)
After 24 hours	99	54 (54%)	46 (46%)

Thus if the Scottish provisional bacteriological standards are adopted and the presence of coliform bacilli in 0.01 ml. of the sample constitutes a failure, 19.7% of these samples failed when tested immediately

after being melted, 27.5% failed when tested 6 hours after melting, 56.4% after 18 hours and 54% after 24 hours.

During the pre-testing period the samples were maintained at a temperature of 20°C.
A comparison of the results of the methylene blue test with those of the presumptive test for coliform bacilli is summarised in the following Table:-

Methylene Blue Test	Samples	Presumptive test for Coliform Bacilli tested			
		Immediately	after 6 hours	after 18 hours	after 24 hours
	per cent	per cent	per cent	per cent	per cent
Passed	68	80.3	72.5	43.5	46
Failed	32	19.7	27.5	56.4	54

It will be seen that the closest correlation between the results of the two tests occurred when the coliform test was begun after the samples had been held at atmospheric temperature for 6 hours. The methylene blue test appears to be rather more stringent than the coliform test at that time, since 32% of the samples failed the methylene blue test and 27.5% the coliform

test. But the dye reduction test was much less exacting than the coliform test carried out on samples 18 hours and 24 hours after melting.

When the coliform test was carried out immediately after the samples were melted, the percentage number of failures was 19.7%, a figure too low to be compared with the grading bases on the methylene blue test.

As with the plate count test it may be that the organisms are"attenuated"to a certain extent by the low temperatures to which the ice cream is subjected and that a pre-testing period at atmospheric temperature renders the test more selective by allowing such organisms to regain their vitality. Since the purpose of the test is to determine the standard of hygiene under which the ice cream is manufactured and also the efficacy of the pasteurisation process, it is important that the test should be capable of revealing as far as possible all visible coliform bacilli.

The fairly close correlation between the results of the methylene blue reduction test and those of the presumptive coliform test after 6 hours is similar to that found between the results of the methylene blue reduction test and the plate counts of the samples after 6 hours at atmospheric temperature, and suggests

that closer agreement and uniformity of standards between the two methods of testing would be obtained if a pre-testing period of 6 hours were allowed before the plate count and presumptive test for coliform bacilli were commenced.

Sub-section 5: Relative Values of the Three Tests - Plate Count, Presumptive Test for Coliform Bacilli and the Modified Methylene Blue Reduction Test - in Ice Cream Plant Control.

The relative significance of the three principal tests used in the routine testing of ice cream is shown in the following case.

Ice cream from a certain manufacturer tested by the methods approved by the Department of Health for Scotland had been showing the presence of coliform organisms in 0.01 ml. of the sample. The plate count on the other hand was low. The modified methylene blue test which was also done resulted in a grade 1 or 2 result.

Three samples tested at intervals gave the following results :-

	Plate count per ml.	Coliform Bacilli per 0.01 ml.	Methylene Blue Reduction Test		
			Hours	Grade	
(1)	10,000	+++	4	2	
(2)	400	+--	5	1	
(3)	9,000	+++	4½	1	

From the results of the plate count alone the samples would have been considered satisfactory in every case. The presumptive test for coliform bacilli however suggested that contamination was occurring at some stage during the manufacturing process. The methylene blue grading was good but from the time taken to reduce methylene blue it was observed that the samples only just reached Grade 1 in examination (2) and (3). Compared to a reduction time of 7½ hours or more, which is so often the case with highly satisfactory samples, these three only just passed the test. The methylene blue test therefore confirmed the impression given by the coliform reaction that there was contamination, though probably only slight (since the plate count was so low) occurring at some stage of the manufacture.

In order to detect the source of contamination samples were taken from the following points in the plant.

(1) The Pasteuriser.

(2) Top of the cooler.

(3) On entering the ageing vat.

Following day.

(4) From ageing vat.

(5) From the continuous freezer.

(6) From the continuous freezer (in carton.)

TABLE XI.

Showing the relative value in detecting contamination of ice cream plant of the plate count and presumptive test for coliform bacilli carried out before and after holding the samples at 20°C for 18 hours and the Methylene Blue reduction times and resulting grades.

Sample taken from	Tested immediately		Tested after 18 hours at 20°C		Methylene Blue Reduction Test	
	Plate count per ml.	Coliform bacilli per 0.01 ml.	Plate count per ml.	Coliform bacilli per 0.01 ml.	Time	Grade
Pasteuriser	1,500	- - -	1,000	- - -	7 hours	1
Top of Cooler	600	- - -	12,000	+ - -	5½ hours	1
On entering ageing vat	2,400	+ + +	65,000	+ + +	5 hours	1
From ageing vat (following day)	1,000	+ + +	38,000	+ + +	5¾ hours	1
From continuous freezer	3,500	+ - -	200,000	+ + +	5½ hours	1
From continuous freezer (carton)	1,200	+ + -	190,000	+ + +	5 hours	1

The plate count and coliform tests were carried out immediately on arrival of the samples in the laboratory and again after they had been held at 20°C for 18 hours. The methylene blue test was carried out in the approved manner and the results of all the tests were compared in order to ascertain which were most effective in detecting the sources of contamination.

The results are given in Table XI.

It will be seen that the sample taken from the pasteuriser passes all the tests. The plate count both before and after holding at 20°C for 18 hours is very low, no coliform organisms are present and the methylene blue is only reduced after 7 hours, so that the sample falls easily into Grade 1.

On passing to the top of the cooler however there is a slight fall in the quality of the product. This is shown by (1) the reduction time being reduced to 5½ hours, and (2) the plate count after 18 hours at 20°C, rising to 12,000 and (3) coliform bacilli being present in one of the three tubes of MacConkey medium when tested after 18 hours at 20°C. The plate count and coliform tests on the other hand carried out immediately give no indication that contamination has taken place.

The sample of ice cream taken as it enters the ageing vat shows a more marked degree of contamination. This is indicated by the coliform tests both before and after the pre-testing period at 20°C. Since all three tubes of the tests are "positive" the sample would be considered to have failed. The plate count on the other hand is still very low on first testing. After the pre-testing period of 18 hours at 20°C however it has increased considerably but is still within the limit of the provisional standard of not more than 100,000 organisms per ml. The grading based on the methylene blue test too is satisfactory but since the methylene blue was reduced in 5 hours it is less satisfactory than the first or second sample and this lowering of the reduction time seems to be significant when taken in conjunction with the results of the coliform test.

The sample taken from the ageing vat on the following day (i.e. after being maintained overnight at a temperature of not more than 45°F) passes all the tests except the coliform tests before and after the pre-testing period. The plate counts have decreased in both tests and the reduction time has increased by ¾ hour. The contamination by coliform organisms is still indicated in all tubes.

From the ageing vat the ice cream mix then passes to the continuous freezer. The sample taken at this point passes the three recognised tests, viz. the plate count and coliform test carried out immediately after the sample was in the frozen state and it also passes the methylene blue test. Such a sample taken singly would be considered entirely satisfactory except that one tube of the coliform test was positive and the methylene blue reduction time was less than is often the case with highly satisfactory samples. The contamination which we know from the previous samples has occurred is only shown up by the plate count and coliform tests carried out on the sample after 18 hours at 20°C, the plate count exceeding the maximum count allowable and the coliform reaction being positive in all three tubes. There is no significant difference between the total counts (when tested immediately) of the samples taken from the ageing vat and from the continuous freezer, but there is a drop in the number of tubes showing a "positive" coliform reaction. This may be due to the effects of the freezing process. Contamination occurring at this stage might not be shown up by the tests carried out so soon after freezing. The plate count of the sample from the continuous

freezer tested after 18 hours at 20°C shows a very marked rise on the previous sample and the test for coliform bacilli is once more "positive" in all tubes. In view of the fact that the grading by the methylene blue test is high it is possible that the count of 200,000 per ml. is an unjustifiable poor result due to the fact that, as has already been pointed out, a "pre-testing" period of 18 hours is too long. Nevertheless a possible source of contamination is shown up in this way. Whether the three tubes showing a positive coliform reaction are due to fresh contamination or to that which occurred earlier in the process it is not possible to say. The increase in the result after the "pre-testing" period may be due to revival of the organisms after a period of dormancy caused by the freezing. The slight decrease in the methylene blue reduction time is not sufficient for any definite conclusion to be drawn from it. Taken together however these three results suggest a minor source of contamination at this stage which is not shown up by the plate count and coliform tests carried out immediately after the sample was taken from the continuous freezer. The results of the tests on the sample in the carton taken from the freezer are almost identical with the results of the

previous sample, but the coliform reaction is positive in two tubes at the time of the first testing and the reduction time is less by half an hour.

To summarise these results, it seems that for plant control work the presumptive test for coliform organisms carried out after a pre-testing period at 20°C is the most effective in showing up possible sources of contamination in ice cream plant. The methylene blue reduction times if a series of samples is tested will also show up any deterioration in quality of the product even though the grade may remain high. The coliform test carried out immediately after heating or cooling is effective to a lesser extent in showing up contamination.

The plate count carried out immediately after heating or freezing of the product is of little value in discriminating between the different samples, but after holding the sample for a period at 20°C the test will show an increase at the points where contamination may have occurred. It is thought however that a "pre-testing" period of 18 hours is probably too long and may give an unjust picture of the degree of the contamination, if a count of not more than 100,000 orgs./ml. is taken as the standard to which all samples should conform.

Sub-section 6: Additional Experiments for determining the most sensitive Test for detecting Contamination of Ice Cream.

INTRODUCTION:

As a result of previous observations it has been suggested by the writer that bacterial plate counts carried out on samples of ice cream, immediately after it has been in the frozen state, may fail to detect any contamination which may have occurred after heat-treatment, from the use of unsterile freezers or containers. The following investigations were carried out in order to determine whether this was in fact the case and if so, which method of examination might be the most likely to detect contamination of this nature.

1. Method :- The ice cream sample, received frozen, was divided into two portions, one of which was placed in a sterile food sample jar and the other in a similar jar which had been rinsed but not sterilised. The two jars containing the ice cream were then placed in a refrigerator set below 32°C for 18 hours. After that time, the portion of ice cream was removed from the unsterile jar and placed in a sterile one. (This corresponds to what should happen during actual sampling.) Both samples were then allowed to melt at room temperature and the plate count and presumptive test for

coliform bacilli were set up, together with the modified methylene blue reduction test. The remainders of each of the portions were then held at 20°C for 18 hours and the plate counts and presumptive coliform tests repeated after that time.

The results of the tests are shown in Table XII., 1.

It is seen that the plate counts of the samples immediately after they had been frozen were so low in both cases, that no conclusions could be drawn from them, regarding the presence or absence of contamination. After 18 hours at 20°C, however the difference between the counts of the sterile and unsterile container is more marked, although the count of the portion from the unsterile jar is well below the maximum allowable and suggests only a mild degree of contamination, this is also borne out by the methylene blue reduction time which was 5½ hours for the contaminated sample, i.e. 1 hour less than for the sample in the sterile jar.

The test was repeated a number of times as described above, but in each case, the unsterile jar was deliberately contaminated in the laboratory by filling with undesignated milk and incubating for 3 hours at 37°C. The milk was then poured out and the jar cooled in the refrigerator for 24 hours. It was then rinsed

TABLE XII.

Showing the relative value of the plate count and presumptive test for Coliform bacilli before and after a pre-testing period at 20°C and the Methylene blue reduction test in detecting contamination of ice cream after Heat-Treatment.

Ice Cream Samples added to sterile and unsterile jars.	Tested immediately after freezing		Tested after 18 hours at 20°C		Methylene Blue Reduction	
	Plate Count per ml.	Coliform orgs. in 0.01 ml.	Plate Count per ml.	Coliform orgs. in 0.01 ml.	Time (hours)	Grade
1. Sterile jar	100	---	1,000	---	6½	1
Unsterile jar	250	---	15,000	---	5½	1
2. Sterile jar	2,600	---	2,600	---	7½	1
Unsterile jar	8,000	---	10,000	---	1½	3
3. Sterile jar	2,100	---	9,500	---	8	1
Unsterile jar	3,200	---	160,000	---	3½	2
4. Sterile jar	7,000	---	Numerous pin point colonies	+++	4	2
Unsterile jar	9,000	---	Uncountable	+++	½	3
5. Sterile jar	1,500	---	6,500	---	7½	1
Unsterile jar	480,000	---	Uncountable	---	0	4

out once with tap water before the addition of half of a sample of ice cream, the other half being placed in a sterile jar. Both jars were then placed in a refrigerator at a temperature of less than 32°C for 18 hours. The contaminated portion was then transferred to a sterile jar and the tests carried out as in Method 1. The results are shown in Tables XII., 2, 3, 4 and 5.

In experiments 2 and 3 there was no significant difference between the plate counts of the ice cream in the sterile jars and those in the unsterile ones, when the tests were set up immediately after the samples were in a frozen condition. After 18 hours at 20°C there was little change in the plate counts in experiment 2 and no indication is given that contamination has taken place from the unsterile jar. In experiment 3, however, the count has risen to over 160,000 for the portion in the unsterile jar and therefore shows up the contamination which was undetected by the earlier count.

The time required to reduce methylene blue by both samples from the unsterile jars was considerably less than by the "sterile" samples. This is particularly noticeable in experiment 2 where the methylene blue reduction test is the only one of the three tests

to show that contamination has taken place, the grading being lowered from 1 to 3 as a result of it. In experiment 3 the methylene blue reduction time was reduced from more than 8 hours to 3½ hours with a resultant lowering of the grade from 1 to 2.

In experiment 4, as in the previous experiments the difference between the plate counts of the two portions, tested immediately after being frozen is of little significance and no coliform bacilli were present in 0.01 ml. of either of them. After 18 hours at 20°C, the plate count of the "sterile" portion had increased to an uncountable number of pin-point colonies, coliform bacilli were present in 0.01 ml. in three tubes and the methylene blue reduction time was 4 hours with a resulting grading of 2, a grading which seems to be lenient for a sample undoubtedly contaminated by thermophilic ~~xx~~ organisms, or organisms slow to develop under the conditions of the test, as well as by coliform bacilli. The plate counts of the portion from the contaminated jar were similar to those of the one from the sterile container, but the methylene blue reduction time was less than 1 hour, resulting in a grading of 3. Thus the methylene blue test has again indicated that additional contamination of the ice cream has occurred.

In experiment 5, contamination from the unsterile jar was sufficient to produce a high plate count even when the sample was tested immediately after being in a frozen condition, as well as when tested after 18 hours at 20°C. The methylene blue reduction time was reduced to 0 hours and the grade lowered from 1 to 4.

Summary:

1. In order to determine the best method of detecting contamination after heat-treatment, samples of ice cream were divided into two portions, one of which was placed in a sterile and the other in an unsterile jar. Plate counts were carried out on the separate portions after all-night refrigeration and again after they had been allowed to stand for 18 hours at 20°C. Methylene blue reduction tests were also carried out in the manner recommended in the English Ice Cream Regulations.

2. The results appear to indicate that slight contamination following heat-treatment will not be observed when the samples are tested by the plate count method immediately after being in the frozen state. When tested after 18 hours at 20°C, on the other hand, contaminated samples are more readily distinguished from uncontaminated ones.

3. Highly contaminated samples may be detected by a

plate count test, carried out immediately after they have been in a frozen state, but this may not be so, where contamination is due to thermoduric, thermophilic or other organisms which have survived pasteurisation and are slow to develop, either as a result of the conditions of the test or because of their "attenuation" due to the heating and cooling processes. One such instance is apparent in the above results, where a sample uncontaminated by laboratory procedure gave a low plate count on first testing, but revealed large numbers of pin-point colonies when tested after a pre-testing period at 20°C. This sample was graded 2 by the methylene blue reduction test.

4. All cases of laboratory contamination of samples were revealed by a lowering of the methylene blue reduction time whether this contamination was slight or of a high degree.

5. From the above observation it appears that the most sensitive test for contamination of ice cream samples is the methylene blue test as recommended in the English Ice Cream Regulations.

The plate count test carried out on samples which have been held for a period of time at atmospheric temperature, or at a controlled temperature of 20°C, is also

of value in detecting contamination unrevealed by a similar test, carried out immediately after the samples have been in a frozen state.

SECTION II.

SYSTEMATIC BACTERIOLOGICAL EXAMINATION OF ICE CREAM.

SECTION II.

SYSTEMATIC BACTERIOLOGICAL EXAMINATION OF ICE CREAM.

INTRODUCTION:

In order that further information regarding the relative values of the different methods of examining ice cream samples might be obtained, it seemed necessary to have some knowledge of the types of bacteria commonly present in ice cream samples, both of high quality supplies and of those where the conditions of production and storage must be considered questionable. With this information, it might then be possible also to determine whether any one species of bacteria present in small numbers could cause unjustifiably low grading of the sample by the modified methylene blue reduction test, for example the thermoduric, aerobic spore-forming bacilli or whether numbers alone irrespective of the species of bacteria are mainly responsible for the reduction of the dye. In this connection, use was made of the Lecitho-Vitellin (L.V.) reaction of McGaughey and Chu (1948) for identifying Bacillus cereus, in order to determine the prevalence of the organism in ice cream and whether it might be found in grades 1 and 2 samples as frequently as in those of grades 3 and 4. A short

sub-section showing the results of the test applied to ice cream samples is included.

The main part of this section is devoted to a systematic study of the bacteria in ice cream, but special attention has been given to the coliform group of organisms, in order not only to determine the type predominating in ice cream, but also to assess the significance of the presumptive test for coliform bacilli as compared with the same test used for the routine examination of drinking water and milk samples.

Sub-section 1: Identification of Bacillus cereus in Ice Cream by means of the Egg-Yolk Reaction of McGaughey and Chu.

One of the criticisms levelled against the modified methylene blue reduction test as a routine method for examining the hygienic quality of ice cream, is that certain spore-forming bacilli, particularly Bacillus cereus, may survive heat-treatment and bring about rapid reduction of methylene blue even when present in only small numbers, and thereby cause unjustifiably low grading of the samples. It seemed that it would be of value to determine what percentage of ice cream samples contained these organisms at the time when the methylene blue was completely reduced, and whether they were as common in samples of Grades 1 and

2 as in Grades 3 and 4.

McGaughey and Chu (1948) showed that B. cereus, B. mycoides and to a lesser extent B. anthracis, are able to produce turbidity and curd formation in egg-yolk saline or broth, due to the action of an enzyme, lecithinase, which is similar to the alpha-toxin of Cl. welchii. The action of the enzyme on the lecitho-vitellin in egg-yolk - known as the L.V. reaction - was shown to be useful for the rapid identification of B. cereus and B. mycoides in mixed cultures.

In order, therefore, to determine whether samples of ice cream contained these organisms, 2 tubes of nutrient broth containing 5% (w/v) egg-yolk were inoculated with 1 loopful of the methylene blue-ice cream mixture after complete reduction of the methylene blue had taken place. One tube was incubated at 37°C and the other at 20°C and were examined periodically up to 6 days for curd production. At 37°C, the curd had usually begun to be formed within 18-24 hours while at 20°C it took from 36 - 48 hours to appear. Subcultures were made from "positive" tubes on to egg-yolk plates which were incubated at 37°C. Colonies surrounded by wide opaque zones of lecithinase reaction were picked and pure cultures of the organisms prepared

and identified according to the method of Gibson and Topping (1938) for identifying aerobic spore-forming bacilli; B. cereus was identified by its colony appearance; acid production in glucose broth; positive Voges-Proskauer reaction; spores producing no swelling of the organism; presence of globules, in the bacilli, unstainable with fuchsin; positive motility; and rapid liquefaction of gelatine.

RESULTS:

Twenty-three samples of ice cream were examined for lecithinase-producing organisms. The methylene blue reduction test had graded these samples as follows: 16, grade 1; 2, grade 2; 2, grade 3; 3, grade 4. The results of the L.V. reaction in egg-yolk broth and the isolation of L.V. positive organisms on egg-yolk agar and their identification are shown in the following Table:-

Grade	Number of Samples Total	Showing L.V. reaction +	Showing L.V. reaction -	from which L.V. + orgs. isolated	from which B. cereus isolated.
1.	16	12	4	12	10 (+ ? 2)
2.	2	1	1	1	1
3.	2	2	0	2	1
4.	3	1	2	0	0
	23	16	7	15	12 (+ ? 2)

B. cereus was isolated from 12 of the 16 grade 1 samples, (in 2 cases, the organisms gave negative Voges-Proskauer reactions but were in other respects, identical with B. cereus) and from one grade 2 and one grade 3 sample.

One lecithinase-producing organism which was not a spore-former was isolated from one of the grade 3 samples. It was gram-negative and although growth occurred at 37°C, the organism was very inactive biochemically at that temperature, no "sugars" were fermented and the L.V. reaction was negative, both in yolk-broth and round the colonies on yolk-agar. At 22°C, however glucose-peptone water was fermented, litmus milk was slowly peptonised and the L.V. reaction became rapidly and strongly positive. The organism was motile and slowly liquefied gelatine. From its characteristics, the organism appears to be a species of the genus Achromobacter.

Although the number of samples of grades 2, 3 and 4 tested is too small for any conclusions to be drawn from the results, the fact that so many of the grade 1 samples had B. cereus present at the time of reduction of the methylene blue, suggests that these organisms are frequently present in ice cream samples but that their presence, in small numbers at least,

will not lower the grading of an otherwise satisfactory sample. Of the 7 samples of grades 2, 3 and 4, B. cereus was isolated twice. In 3 the L.V. reaction was quite negative and this may be due to inhibition by other organisms present in the samples.

Further information regarding the presence of B. cereus in ice cream is given in the following subsection which deals with the complete bacterial flora of 11 samples of ice cream.

Sub-section 2: Bacterial Flora of Ice Cream.

Eleven samples of ice cream were examined systematically before and after reduction of methylene blue. Quality of the samples:

When tested immediately after being in the frozen state, the plate counts varied from 150 per ml. to "uncountable". After 18 hours at 20°C the counts ranged from 500 per ml. to "uncountable". If the provisional standard of not more than 100,000 organisms per ml. was applied to the samples when tested immediately, one of the eleven would fail. If applied after the samples had been held at 20°C for 18 hours before testing, four would fail.

The grading based on the results of the methylene blue test was as follows:

Grade 1 :- 5 samples.

Grade 2 :- 2 samples.

Grade 3 :- 2 samples.

Grade 4 :- 2 samples.

Thus four of the eleven would be considered unsatisfactory according to the results of the methylene blue test. This figure corresponds to the number which failed the plate count standard when tested after 18 hours at 20°C.

TABLE XIII.

Showing the Results of Tests carried out on 11 Samples of Ice Cream, and the Organisms isolated from them, before and after Reduction of Methylene Blue.

Ref. No.	Plate Counts of Samples Tested		Coliform Bacilli in 0.01 ml. Samples Tested		Meth. blue Reduction		L.V. Reaction Before Red^{n} of Meth. Blue	L.V. Reaction After Red^{n} of Meth. Blue	Organisms Isolated from Ice Cream	
	Immed. (thou./ml.)	After 18 hrs. (thou./ml.)	Immed.	After 18 hrs.	Time (hrs)	Grade			Before Red^{n} of Meth. blue	After Red^{n}
53.	70.0	Unc.	---	+++	1½	3	+	+	Strep. faecalis Achromobacter delicatum Flavobacterium diffusum B. cereus	
54.	0.45	50.0	---	+--	>8	1	-	+	No growth	Alcaligenes metalcaligenes Paracolobactrum aerogenoids
55.	0.6	26.0	---	---	3½	2	+	+	Strep. faecalis	Achromobacter liquefaciens Alcaligenes bookeri Aerobacter cloacae
56.	17.5	Unc.	---	+++	0	4	(+)	-	Sarcina sp. Corynebacterium sp. Aerobacter cloacae Paracolobactrum aerogenoids	
57.	1.4	30.0	---	++-	1½	3	+	+	Achromobacter sp. B. cereus.	
58.	0.15	0.5	---	---	7	1	-	+	B. circulans	B. subtilis B. cereus
68.	0.9	2.0	---	---	6½	1	-	(+)	Micrococcus pyogenes albus	Esch. intermedium B. cereus
69.	5.0	7.0	---	+++	6	1	-	+	Micrococcus sp. Alcaligenes metalcaligenes	Paracolobactrum aerogenes B. cereus
70.	60.0	170.0	+++	+++	3-5	2	+	+	Alcaligenes faecalis radicans B. cereus	Aerobacter cloacae Alcaligenes faecalis
71.	7.5	45.0	---	---	7½	1	-	+	Strep. faecalis Corynebacterium sp. Alcaligenes metalcaligenes	Esch. intermedium Aerobacter aerogenes B. cereus
72.	Unc.	Unc.	+++	---	0	4	+	(+)	Strep. faecalis Esch. coli B. cereus	

Abbreviations:- Immed. = Immediately. Meth. blue = Methylene blue. Red^{n} = Reduction Unc. = Uncountable
thou. = Thousands > = more than + = positive reaction.
hrs. = hours L.V. = Lecitho-vitellin reaction (+) = weak positive reaction.

Isolation and Identification of Bacteria :-

1. Blood agar and MacConkey plates and a tube of yolk-broth for the lecithinase test for B. cereus identification, were inoculated with a loopful of the ice cream and at the same time a film of the sample was prepared and stained by Gram's method.

2. After completion of the methylene blue test, similar inoculations were made with the reduced methylene blue ice cream mixture and a film prepared and stained by Gram's method.

3. A loopful of each yolk-broth culture giving a positive lecithinase reaction after incubation for 2 or 3 days was plated out on yolk agar and representatives of those colonies surrounded by the characteristic opaque zones caused by lecithinase reaction were isolated and identified.

The results of the various tests carried out on the eleven samples are summarised in Table XIII., from which the bacterial flora of samples of different grades may be compared.

Five samples were grade 1, and the L.V. reaction of all five was negative when the yolk-broth was inoculated directly from the ice cream. If on the other hand the reduced methylene-blue-ice cream mixture was tested,

the reaction was positive in every case. This suggests that B. cereus was present in all the samples but in no case was it in sufficient numbers to be detected by the L.V. reaction until after a period of time had elapsed, viz. in these samples the period sufficient to allow reduction of the methylene blue by the organisms in the ice cream.

The distribution of the micro-organisms isolated and identified was as follows, Bergey's classification being used throughout. The numbers in brackets (1) and (2) after the species indicates whether the organism was isolated, (1) directly from the ice cream or (2) after reduction of the methylene blue. The reactions of the various organisms are shown in Tables XIV. and XV.

Grade 1 samples:-

1.	Alcaligenes metalcaligenes	(2)
	Paracolobactrum aerogenoides	(2)
2.	B. subtilis	(2)
	B. cereus	(2)
	B. circulans	(1)
3.	Micrococcus pyogenes albus	(1)
	Escherichia intermedium	(2)
	B. cereus	(2)
4.	Micrococcus sp.	(1)
	Alcaligenes metalcaligenes	(1)

TABLE XIV.

Showing the Biochemical Reactions of a number of Organisms isolated from Ice Cream including some Unidentified Species. (Members of Family Achromobacteriaceae are described in Table

Ref. No.	Gram Reactn.	Organisms	Glucose	Lactose	Sucrose	Dulcitol	Mannitol	Starch	Glycerol	Litmus milk	Gelatine liquefn.	Voges Proskauer	Methyl Red	Indole	Citrate Utiln.	Red.n of Nitrates	Motility	Eijkman Reactn.	Growth on agar	Growth on MacC.	Identified
54(1),(4)&(5)	-	bacillus	SlAG	-	A	-	A	A	SlAG	A	-	+	.	+	+	+	+	-	Grey	Pale becoming Pink	Paracolobactrum aerogenoides
56(2)	+	cocci (tetrads)	SlA	-	-	-	-	SlA	.	SlA	-	-	.	.	.	-	-	.	Yellow	.	Sarcina sp.
"(3)	+	bacilli	A	.	A	.	.	A	.	.	+	+	.	.	.	-	-	.	Colourless	.	Corynebacterium sp.
" (4)	-	bacilli	SlAG	-	SlAG	-	SlAG	SlA	SlAG	A	-	+	.	.	.	+	+	.	Grey	Pale becoming Pink	Paracolobactrum aerogenoides
57(2)&(3)	-	bacilli	A	-	-	-	-	.	.	A clot	+	-	.	-	.	-	-	.	Grey	-	Unidentified sp.
68(4)	+	cocci	A	A	-	-	A	-	SlA	SlA	+	-	-	.	.	+	.	.	White	White	Micrococcus Pyogenes albus
69(1)&(2)	-	bacilli	AG	SlAG	AG	-	AG	A	SlA	A	-	+	-	-	+	+	+	-	Grey	Pale becoming Pink	Paracolobactrum aerogenoides
"(3)	+	diplo-cocci	-	-	-	-	-	-	.	A	+	-	-	-	-	-	-	-	Deep yellow	.	Micrococcus sp.
71(6)	+	bacilli	A	-	A	-	-	-	.	Alk	-	-	+	-	+	-	-	.	Buff	.	Corynebacterium sp.

\+ = positive reaction; - = negative reaction.
A = acid; AG ; acid and gas; alk. = alkaline reaction.
SlA = late or weak reaction
SLAG = " " " "

TABLE XV.

Showing the Biochemical Reactions of Gram-negative Organisms forming pale colonies on MacConkey plates, isolated from Ice Cream and identified as members of Family "ACHROMOBACTERIACEAE".

Ref.No.	Glucose	Lactose	Sucrose	Dulcitol	Mannitol	Starch	Glycerol	Litmus milk	Gelatin liqueⁿ.	V.P.	M.R.	Indole	Citrate Utilⁿ.	Redⁿ. of Nitrates	Motility	Growth on agar	Identification
53(2)	A	-	-	-	-	-	Sl.A	Sl.A	+	-	-	-	+	+	+	Colourless	Achromobacter delicatum.
" (6)	A	-	A	-	A	A	-	-	+	-		-		+	+	Yellow	Flavobacterium diffusum.
54(2) & (3)	-	-	-	-	-	-	Sl.A	Alk.	-	-		-		-	-	Colourless	Alcaligenes metalcaligenes.
55(1),(2) & (4)	A	-	-	-	-	A	Sl.A	Sl.A	+	-		-		-	+	Cream	Achromobacter liquefaciens.
" (7)	-	-	-	-	-	-		Redⁿ.	+	-	-	-	-	-	+	Grey	Alcaligenes bookeri.
69(5)	-	-	-	-	-	-	Sl.A	Alk.	-	-	-	-		-	-	Cream	Alcaligenes metalcaligenes.
70(2)	-	-	-	-	-	-	A	Alk.	+	-	-	-		±	+	Cream	Alcaligenes faecalis radicans.
" (3)	-	-	-	-	-	-	A	Alk.	-	-	-	-		-	+	Cream	Alcaligenes faecalis.
71(5)	-	-	-	-	-	-	Sl.A	Sl. Redⁿ.	-	-	-	-		-	-	Colourless	Alcaligenes metalcaligenes.

+ = positive reaction; - = negative reaction.
A = Acid; Alk = Alkaline; redⁿ = reduction.
Sl.A = late or weak reaction.
Sl. Redⁿ. = " " "

148.

	Paracolobactrum aerogenoides	(2)
	B. cereus	(2)
5.	Streptococcus faecalis	(1)
	Corneybacterium sp.	(1)
	Alcaligenes metalcaligenes	(1)
	Escherichia intermedium	(2)
	Aerobacter aerogenes	(2)
	B. cereus	(2)

It is seen that a fairly wide variety of organisms was isolated from Grade 1 samples, but in most cases only after reduction of the methylene blue, either directly from the methylene blue-ice cream culture or from a yolk broth culture giving a positive L.V. reaction. In view of the fact that B. cereus was isolated from 4 out of 5 of the grade 1 samples and was probably present in the fifth as indicated by the positive L.V. reaction, and by the presence of Gram-positive bacilli in direct films of the reduced methylene blue-ice cream mixture, it seems unlikely that its presence alone in small numbers could have accounted for the low grading of ice cream as reported by various critics of the methylene blue test. None of the five grade 1 samples was a "border-line" case, all requiring 6 hours or more to bring about reduction of methylene

blue and one failing to do so in 8 hours. In one sample with a reduction time of 7 hours, aerobic spore-forming organisms, only were isolated. They were identified as B. subtilis, B. cereus and B. circulans.

The greatest variety of organisms was isolated from one sample which required 7½ hours to reduce methylene blue. In spite of its high grading on the basis of the methylene blue test and its low bacterial count, it was not entirely satisfactory since Streptococcus faecalis was isolated from it in direct culture, and was seen in the direct film. This organism which is heat-resistant had presumably withstood the temperature of pasteurisation but was not in sufficient number to give an unsatisfactory result in any of the recognised tests.

Grade 2 samples:-

1.	Streptococcus faecalis	(1)
	Alcaligenes bookeri	(2)
	Achromobacter liquefaciens	(2)
	Aerobacter cloacae	(2)
2.	Alcaligenes faecalis radicans	(1)
	Alcaligenes faecalis	(2)
	Aerobacter cloacae	(2)
	B. cereus	(1)

As with the Grade 1 samples, many of the organisms from the two Grade 2 samples, were isolated only after the period of time had elapsed, necessary to bring about reduction of methylene blue. B. cereus was present in both samples, as evidenced by the positive L.V. reactions and the Gram-positive bacilli, seen in the direct films, and it was isolated in pure culture from one of them. Aerobacter cloacae and species of Alcaligenes were also present in both. Streptococcus faecalis was isolated directly from one of the samples. It seems unlikely that the sporing organisms were wholely responsible for lowering the grades of the samples to 2. In one sample, although the plate counts were well within the prescribed limit, and the coliform reaction was negative, Streptococcus faecalis and Aerobacter cloacae were isolated, and these might be just as responsible for lowering the reduction time as B. cereus.

Grade 3 samples:-

1.	Streptococcus faecalis	(1)
	Achromobacter delicatum	(1)
	Achromobacter sp.	(1)
	Flavobacterium diffusum	(1)
	B. cereus	(1)

2. Achromobacter sp. (1) and (2)

B. cereus (1)

It is noticeable that all the species listed above were isolated from the Grade 3 samples directly from the ice cream, so that they were presumably present from the start in fairly high numbers. Streptococcus faecalis was again isolated from one of them, and B. cereus from both. In one, the plate counts were low and B. cereus and species of Achromobacter were the only organisms isolated. Since species of Achromobacter have been found to be very inactive in reducing methylene blue, it is probable that the reduction in this case was mainly due to B. cereus, but the coliform reaction was positive if a pre-testing period of 18 hours at 20°C was allowed, so that here again the sporing organisms might not be entirely responsible for the fairly rapid reduction of the methylene blue.

Grade 4 samples:-

1. Aerobacter cloacae (1) and (2)

Sarcina sp. (1)

Corynebacterium sp. (1)

Paracolobactrum aerogenoides (1)

2. Streptococcus faecalis (1)and (2)

Escherichia coli (1)and (2)

Bacillus cereus (1)and (2)

All the species listed above were isolated from the Grade 4 samples directly from the ice cream, so that they were presumably present from the start in fairly high numbers.

Streptococcus faecalis was present along with typical B. coli in one sample which had an uncountable number of colonies present on the agar plates. B. cereus was also present.

The other sample had a low plate count in the first instance but numerous pin point colonies were present in addition to a count of 64,000 after a pre-testing period. Coliform organisms were also present but were detected only at the time of the second testing. No aerobic spore forming organisms were isolated. It seems that, although the majority of organisms were not able to grow readily under the conditions of the plate count test, the total bacterial content of this sample was high,and that one or more of the four species isolated from the sample may have been rapid reducers of methylene blue.

It is shown in a later section (c/f Table that both the Corynebacterium sp. and the Aerobacter cloacae isolated from this sample were active reducers of methylene blue.

Summary:-

(1) Eleven samples of ice cream were examined systematically before and after reduction of methylene blue. They included 5 grade 1 samples; 2 grade 2; 2 grade 3, and 2 grade 4, the grading being based on the methylene blue reduction times.

(2) In the cases of the 5 grade 1 samples, a fairly wide variety of organisms was present, but in the majority they were isolated, only after reduction of the methylene blue, either directly from the reduced methylene blue-ice cream mixture or from a yolk broth culture giving a positive L.V. reaction.

(3) Since B. cereus was detected in all of the 5 grade 1 samples, it seems unlikely that its presence alone in small numbers could have accounted for low grading of ice cream as reported by various critics of the methylene blue test for ice cream.

(4) In the case of the 2 grade 2 samples, B. cereus was present, but since Aerobacter cloacae was also

present in both, and Streptococcus faecalis in one of them, it seems unlikely that the sporing organisms were wholly responsible for lowering the grade of the samples to 2.

(5) All of the 7 species detected in the grade 3 samples and the 7 in the grade 4 samples were isolated directly from the ice cream so were presumably present from the start in fairly high numbers.

6. With the possible exception of one of the grade 3 samples where reduction of methylene blue seemed to be mainly due to the presence of B. cereus, no one species of organism predominated in Grades 3 or 4 which might have accounted for the rapid reduction of methylene blue and there was no indication that the low grading was unjustifiable.

Sub-section 2.

CLASSIFICATION OF COLIFORM BACILLI FROM ICE CREAM.

Introduction:-

Bardsley (1934) in the course of an investigation into the distribution and sanitary significance of coliform bacilli in water, soil, faeces and ice cream, examined 44 samples of ice cream by means of the presumptive test for coliform bacilli. Twenty-seven (61.3%) of them gave a positive reaction and from them were isolated 365 strains of lactose-fermenting organisms. The following tests were then used to identify the different strains :-

(1) Methyl-red reaction.

(2) Voges-Proskauer reaction.

(3) Test for indole production.

(4) Koser's uric acid test.

(5) Koser's citrate utilisation test.

From the results of these five tests the organisms were divided into three sub-groups, viz.:-

Sub-group	M.R.	V.P.	Indole	Uric Acid Util.	Citrate Util.
1. *B. coli*	+	-	+	-	-
2. *B. lactis aerogenes*	-	+	±	+	+
3. Intermediate type.	+	-	-	-	+

She found that organisms with the characteristics of sub-group 2, i.e. B. lactis aerogenes, predominated, accounting for 67% of the total and were present in 81.5% of the ice cream samples. Organisms of the intermediate type accounted for 19% of the total number of strains and were present in 48% of the samples. Only 7% of the strains were identified as B. coli and they were present in 26% of the samples.

Seven per cent of the strains gave biochemical reactions which did not correspond to those of any of the three sub-groups, and these were placed in a separate sub-group, designated "irregular strains".

The predominance of organisms of the B. lactis aerogenes type was considered by Bardsley to be due to their frequent presence in large numbers in the milk and milk products from which the ice creams were manufactured and also to the possibility that these strains are more resistant to the prolonged exposure to the low temperature used in the manufacture of ice cream than are the other members of the group of coliform bacilli.

Bardsley (1938) reports on a further 237 samples of ice cream taken at random from different vendors whose methods and conditions of manufacture varied considerably. She found coliform bacilli in 209

(88%) of them, the number of organisms ranging from 1 to 180,000,000 per 100 ml. of the sample. Two hundred and fifty-one strains were isolated from 65 of the samples and this time they were classified according to the method of Wilson (1935), viz.:-

	M.R.	V.P.	Citrate	Indole	Eijkman 44°C	Gelatine liquefaction
Bact. coli I	+	-	-	+	+	-
Bact. coli II	+	-	-	-	-	-
Intermediate type I.	+	-	+	-	-	-
Intermediate type II.	+	-	+	+	-	-
Bact. aerogenes I	-	+	+	-	-	-
Bact. aerogenes II	-	+	+	+	-	-
Bact. cloacae	-	+	+	-	-	+
Irregular strains	variable					

B. lactis aerogenes (Bact. aerogenes) was again the predominating type and accounted for 45.8% of the total number of strains; 35.8% were classed as intermediate types, 13.9% as Bact. coli and 3.9% were irregular strains.

The proportions of the different types reported in 1934 and 1938 are summarised in the following Table:-

	1934	1938
B. coli (Bact. coli I and II)	7%	13.9%
B. lactis aerogenes (Bact. aerogenes I and II)	61%	45.8%
Bact. cloacae	—	0.39%
Intermediate types	19%	35.8%
Irregular strains	7%	3.9%
Total number of strains examined	365	251

Differentiation of Bact. lactis aerogenes strains into (a) Bact. lactis aerogenes and (b) Bact. cloacae.

No attempt was made by Bardsley (1934) to divide the sub-group then referred to as "B. lactis aerogenes" into different species. Twelve strains which liquefied glucose-gelatine medium after incubation for 5 days at 20°C were discarded. By the use of

Wilson's classification, Bardsley (1938) identified 88 strains as Bact. lactis aerogenes type I, 27 strains as Bact. lactis aerogenes type II and 1 strain as Bact. cloacae. Wilson (1935) differentiated Bact. cloacae from Bact. aerogenes on the basis of the ability of the former to liquefy gelatine medium within 3 weeks at 22°C. He found that of 496 strains isolated from raw and pasteurised milk, cow dung and foodstuffs, 31 Bact. cloacae and 6 irregular strains liquefied gelatine. The majority were slow liquefiers failing to do so within 5 days. Thus according to Bardsley and Wilson Bact. cloacae appears to be of minor importance, accounting for only a small percentage of the total number of coliform organisms. Malcolm (1938) on the other hand recognises four principal sub-groups of coliform organisms, one of which is designated B. cloacae, the others being B. coli, B. aerogenes and B. oxytocus. These four sub-groups are distinguishable from one another by the following four tests :- Voges-Proskauer reaction, Koser's citrate utilisation test, inositol fermentation and indole production, viz.:-

	V.P.	Citrate Util.	Inositol	Indole
B. coli	-	-	-	+
B. cloacae	+	+	-	-
B. oxytocus	+	+	+	+
B. aerogenes	+	+	+	-

B. cloacae strains are therefore distinguishable from B. aerogenes by their inability to ferment inositol. B. oxytocus strains appear to correspond to Wilson's B. aerogenes type II although they may be distinguished from them by their greater fermentative powers and by their ability to liquefy gelatine slowly. Whereas Wilson observed liquefaction or non-liquefaction of gelatine in one tube only, after incubation at 22°C up to a maximum of 3 weeks, Malcolm (1938) carried out the test in duplicate, one tube being maintained at 37°C for 12 weeks, during which time it was periodically examined for liquefaction after placing in cold water for a few hours, and thereafter, after a further 24 hours at room temperature. The other tube was kept at room temperature for 8 weeks and if the gelatine was still undigested it was then incubated at 37°C for 4 weeks. Thus fully three months were allowed to elapse in each case before a negative result was

reported.

Bergey (1948) considers aerogenes and cloacae as separate species of one genus Aerobacter, the distinguishing feature being the ability of Aerobacter aerogenes to produce gas as well as acid in glycerol, whereas Aerobacter cloacae ferments glycerol with no visible gas formation. This key to the species of Aerobacter was based on the work of Kliger (1914) who identified those coliform organisms which ferment saccharose and salicin as B. aerogenes, and then divided the group into two sub-groups viz. (1) those which fermented glycerin and failed to liquefy gelatine, and (2) those which failed to ferment glycerin but were able to liquefy gelatine. The latter he designated B. cloacae, but found that with certain strains the property of liquefying gelatine had apparently been lost. It is not clear how long these cultures were maintained before a non-liquefying result was recorded. It appears that some property other than gelatine-liquefaction is required to combine with the glycerol fermentation test to differentiate cloacae strains from aerogenes.

Griffin and Stuart (1940) attempted to separate the species of Aerobacter on the basis of the results of the three tests:- glycerol fermentation, gelatine liquefaction and motility. They found two main forms of correlation between the three reactions viz.:-

(1) Glycerol fermentation:- acid and gas production.
Gelatine liquefaction:- negative.
Motility:- negative or positive.

(2) Glycerol fermentation:- acid only or negative.
Gelatine liquefaction:- positive.
Motility:- positive.

Out of a total of 94 strains of Aerobacter species, isolated from samples of milk, water, soils, grains and faeces, 53.2% (38.3% motile and 14.9% non-motile) gave reactions which conformed to group (1). They were classed as Aerobacter aerogenes. 30.9% conformed to group (2) and were Aerobacter cloacae. But in addition there were 15.9% which did not show either of the two forms of correlation between the results of the three tests. They were classed as "irregular". In view of this high percentage of strains which failed to conform to the system of classification, Griffin and Stuart expressed doubt as to the advisability of placing aerogenes and cloacae strains

in separate species. An examination of their findings, however, show that if correlation between the results of two out of three of the tests is used to separate the strains, motility being accepted as a characteristic of cloacae strains, then the irregular strains could be divided as follows :-

(1) Aerobacter aerogenes (irregular in being gelatine-liquefiers) :- 9.6% of the total.
Some of these strains might have been identical with B. oxytocus (Malcolm) a species which is similar to type II Aerobacter aerogenes but which is a gelatine-liquefier.

(2) Aerobacter cloacae (irregular in producing acid and gas in glycerol) :- 2.1% of the total.

(3) Aerobacter cloacae (irregular in failing to liquefy gelatine) :- 4.2% of the total.

Had observations of gelatine-liquefaction been continued for longer than 40 days. it is possible that the number of strains of non-gelatine-liquefying cloacae might have been reduced.

Mucoid Colonies, Capsules and Slime Production.

Wilson (1935) studied the appearance of colonies of coliform organisms after growth on MacConkey agar plates at 37°C, observations being

made after 24 hours and 4 days. He found that mucoid colonies were more frequent among aerogenes and cloacae than among coli strains. Whereas 73% aerogenes and 61% cloacae strains produced mucoid growth, only 20% coli strain did so. As shown by Duguid (1951) mucoidness may result either from the growth of capsulated organisms and from loose slime produced by them, or from loose slime only, produced by non-capsulated organisms. Of 40 aerogenes strains examined by him, 37 formed capsules and were abundant slime-producers and 3 were non-capsulated, motile, slime-forming strains. Of 55 coli strains also examined, 2 produced capsules and slime, 8 produced slime only and 45 produced neither capsules nor slime.

The section of experimental work which follows, deals with the identification of a number of strains of coliform organisms, isolated from 53 samples of ice-cream. Use has been made of the differential tests employed by previous workers, viz.:-
(1) Methyl red reaction; (2) Voges-Proskauer reaction; (3) Citrate-utilisation; (4) Indole-production; (5) Production of visible gas at 44°C; (6) Gelatine-liquefaction; (7) Motility; (8) "Sugar"-fermentation reactions including the following "sugars":- glucose,

lactose, sucrose, dulcitol, mannitol, glycerol and inositol. The formation of mucoid colonies as a result of capsulated and slime-producing organisms was investigated as possibly affording additional criteria for differentiating Aerobacter species. This latter group predominates among the coliform bacilli in ice cream and received special attention. A scheme of classification is presented, based on the results of the above tests.

EXPERIMENTAL.

Identification of Coliform bacilli isolated from Ice Cream.

Ninety cultures were isolated from 53 samples of ice cream which had given positive reactions to the presumptive test for coliform bacilli, either when tested immediately on arrival in the laboratory or after a pre-testing period of 6, 12 or 18 hours at a temperature of not more than 20°C. The cultures were studied by various differential tests and identified as far as possible.

Method of Isolation.

Three tubes of MacConkey broth were each inoculated with 1 ml. of a 1 in 100 dilution of the sample and incubated at 37°C for 48 hours. From each tube showing acid and gas, one loopful was plated on MacConkey agar and after 24 hours incubation at 37°C, representatives of all types of lactose-fermenting colonies were picked and cultured on agar slopes. At the same time subcultures were made from positive tubes into fresh MacConkey broth, and these were incubated at 44°C and examined for gas production after 24 and 48 hours. If gas was present the cultures were

plated out on MacConkey agar and suitable colonies picked. All the agar slope cultures so obtained were replated on MacConkey agar and fresh cultures made from single colonies, while throughout the investigation replating was done repeatedly in order that there should be no doubt regarding the purity of the strain under examination.

The ability of the strains to produce acid and gas in lactose-peptone water after incubation at 37°C was confirmed in each case before any of the differential tests were carried out. The nomenclature used is that of Bergey (1948), but methods of classification are based mainly on those of Wilson (1935) with modifications where these have proved to be of value.

Preliminary Classification.

The strains were divided into three groups on the basis of the results of the following tests :-

1. Methyl red reaction after 5 days growth in glucose-phosphate-peptone water at 30°C.
2. Voges-Proskauer reaction after 2 - 5 days growth in glucose-phosphate-peptone water at 30°C (Barratt's modification).
3. Citrate-utilisation test at 37°C (and at 30°C if growth was negative or slow at 37°C.)

Grouping was as follows:-

1. Escherichia:- M.R. +, V.P. -, Citrate utilisation -.

2. Aerobacter :- M.R. -, V.P. +, Citrate utilisation +.

3. Intermediate:- M.R. +, V.P. -, Citrate utilisation +.

The 90 strains examined fell into the groups as follows.

Escherichia:- 9 strains.

Aerobacter:- 57 "

Intermediate:- 24 "

Total 90.

1. Escherichia group.

These strains were further examined for :-

1. Production of indole in peptone water after 48 hours incubation at 37°C.

2. Production of gas in MacConkey broth at 44°C, i.e. Eijkman test.

Of the nine strains examined, all were found to be indole positive and Eijkman test positive. They therefore correspond to Bact. coli type I. Wilson, and to Escherichia coli, Bergey, although the latter species also includes indole negative and Eijkman negative strains. Had any of these been isolated it is suggested that a more suitable designation for them

would be Escherichia coli, type 2, while the nine strains described above, whose reactions are typical of the species should be designated Escherichia coli, type I.

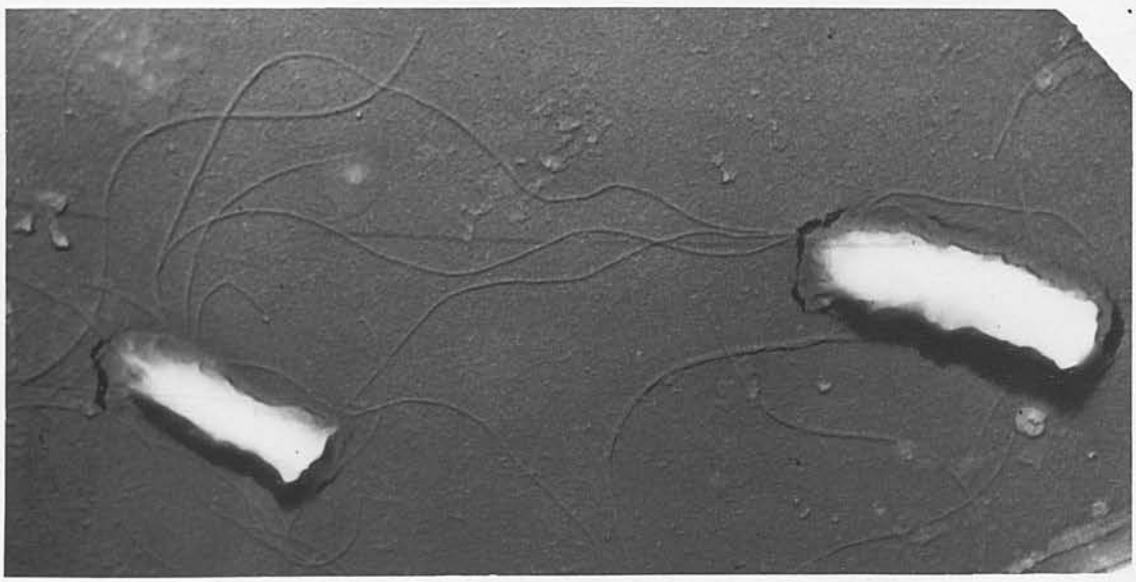
2. Aerobacter group.

As found by Bardsley (1934 and 1938), the members of this group were the predominating type of coliform organism in ice cream, accounting for over 66% of the total. They were differentiated further by means of the following tests:-

1. Liquefaction of gelatine.
2. Glycerol fermentation.
3. Motility.
4. Indole production.
5. Presence of capsules and slime formation.

Gelatine liquefaction:- Tubes of gelatine medium, prepared by dissolving gelatine in neutral broth in the proportion of 10 - 15% depending on the time of year, were heavily inoculated from an agar slope culture by stab culture technique and incubated at 30°C for 48 hours. They were then brought into the Laboratory and kept under observation for up to four months or longer.

Fig. 1.



Aerobacter cloacae, 259 (1), isolated from ice cream, showing flagella.

Magnification x 20,000.

Electron Micrograph by Miss I. W. Smith.

Glycerol-fermentation:- Tubes of peptone water each containing 0.5 ml. glycerol, neutral red to detect acid change and a Durham tube for gas production were inoculated with the culture and examined for acid and gas after 24 and 48 hours at 37°C. If negative they were then kept at laboratory temperature for a week and examined daily.

Motility was tested by examination of hanging-drop preparations of 6 hour peptone water cultures. If negative results were obtained where positive ones were expected, they were confirmed by examination of the culture for flagellae by electron microscopy. The author is indebted to Miss Isobel Smith, B.Sc., for carrying out these confirmatory tests. (See Fig. 1.)

If the Aerobacter group ~~are~~ is divided into aerogenes and cloacae species on the basis of glycerol-fermentation and motility, thus:-

	Fermentation of glycerol.	Motility.
Aerobacter aerogenes:	Acid and gas	Negative
Aerobacter cloacae:	Acid only	Positive

then the 57 strains examined could be divided as follows :-

Aerobacter aerogenes:-	22		
Aerobacter cloacae:-	31		
Irregular strains of Aerobacter	(a)	glycerol:- acid and gas motility:- positive	2.
	(b)	glycerol:- acid only motility:- negative	2.

With regard to gelatine-liquefaction, of the 22 strains of Aerobacter aerogenes, 1 proved to be a gelatine-liquefier, and 6 of the 31 strains of Aerobacter cloacae failed to liquefy gelatine.

Of the 2 irregular strains "a", one liquefied gelatine and this characteristic taken along with its motility might be considered sufficient to identify it as Aerobacter cloacae which was irregular in view of its ability to produce gas in glycerol medium. The other did not liquefy gelatine and this feature along with its ability to produce gas in glycerol, might be sufficient to classify it as Aerobacter aerogenes which was irregular in being motile. This strain was also slow to utilise citrate. The two irregular strains "b" were both gelatine-liquefiers and since both failed to produce gas in glycerol they could be identified as Aerobacter cloacae which were irregular in being non-motile.

Thus if no conclusions can be drawn from the

results of glycerol fermentation and motility tests taken together, the gelatine-liquefaction test may be useful in helping to identify as nearly as possible certain irregular strains.

Indole-production.

Generally speaking, organisms of the Aerobacter group do not produce indole in peptone water cultures. Wilson, however, found that 12% of aerogenes strains examined by him were indole-positive. They were designated Bact.aerogenes, type II. Bergey includes both indole-producing and non-indole-producing strains in his genus Aerobacter. Here it is proposed to separate the two and to name the non-indole-producing strains, Aerobacter aerogenes, type I. and the indole-producers, Aerobacter aerogenes, type II.

Of the 23 strains identified as Aerobacter aerogenes, 4 were indole positive. Three of them were isolated from the same sample so that only 2 out of the 53 samples of ice cream were found to contain Aerobacter aerogenes, Type II.

Without exception all the 34 strains identified as Aerobacter cloacae were indole-negative.

Production of Mucoid Colonies as a result of Capsule and Slime-formation by strains of Aerobacter aerogenes and Aerobacter cloacae.

It has been recognised by previous workers that species of Aerobacter tend to form mucoid colonies when grown on MacConkey lactose agar. This is due either to the presence of capsules round the organisms or, in the case of motile strains, to loose slime-formation. As stated in the introduction to this section, Duguid (1951) has described a method of producing optimum conditions for the production of mucoid colonies by culturing the organisms on excess-sugar peptone agar. His wet-film India-ink technique is a reliable method for determining whether mucoidness is due mainly to the presence of capsules or to purely slime-forming strains. The writer is indebted to him for examining in this way 34 of the Aerobacter strains isolated from ice-cream, duplicate strains from the same samples, identified by previous tests having been omitted. From the results it was hoped that further differential criteria might be obtained for distinguishing aerogenes from cloacae.

The results of previous tests had separated these 34 strains into:(a) 10 Aerobacter aerogenes,

TABLE XVI.

PRODUCTION of MUCOID COLONIES, CAPSULES and SLIME

By Species of Aerobacter.

No.	Mucoid Growth at		Capsules	Slime
	35°C	Room temperature		
(a) Aerobacter aerogenes.		Type I.		
83264 (1)	++	+++	+++	+
83265 (1)	—	+++	+++	+
83889 (1)	—	++++	++++	++
84326 (1)	—	++	++	+
84434 (1)	++	+++	++	+
84438 (3a)	++	+++	++	Slight
85903	—	++	++	Slight
2 (1)	—	++	++	Slight
W (2)	+	+++	++	+++
83921 (2)	—	—	—	—
(b) Aerobacter aerogenes.		Type II.		
87376/2 (1)	—	+++	+	+
84034/2 (1)	—	+++	+	Slight

TABLE XVI. Contd.

No.	Mucoid Growth at		Capsules	Slime
	35°C	Room temperature		
(c) Aerobacter cloacae.		Typical.		
82602 (1)	+++	+++	—	+++
82636 (1)	+++	+++	—	+++
83259 (1)	Slight	+++	—	+++
83919 (1)	—	++	—	+++
83923 (1)	+++	+++	—	+++
83989 (1)	—	+++	—	+++
84034 (1)	++	+++	—	+++
84035 (1)	Slight	+	—	+++
84100	Slight	+	—	+++
84434 (2)	+++	+++	—	+++
84438 (1a)	++	++	—	+++
84793 (1)	+++	+++	—	+++
87265	+++	+++	—	+++
87377/2 (1)	—	+	—	+++
(d) Aerobacter cloacae.		Atypical.		
I. Non motile				
84439 (1)	Slight	++	—	+++
II. Gas formed in glycerol medium.				
82637 (2)	+++	+++	—	+++
III. Non-gelatine-liquefying strains.				
82561 (1)	—	—	—	—
82639 (2)	++	+++	—	+++
83889 (3)	—	++	—	+++
83923 (2a)	—	++	—	+++
83924 (1)	—	+++	—	+++
84035/2 (3)	—	++	—	+++

type I, one of which was atypical in being motile and slow to utilise citrate at 37°C, (b) 2 Aerobacter aerogenes type II, one of which was a gelatine-liquefying strain and owing to its wide fermentative powers appeared to correspond to B. oxytocus, Malcolm, (c) 14 typical Aerobacter cloacae, and (d) 8 atypical Aerobacter cloacae (6 non-gelatine-liquefying, 1 non-motile, and 1 strain which fermented glycerol with gas-formation). The results of the tests for capsules and slime-production are given in Table XVI an examination of which reveals the following points of interest :-

(1) Only 4 of the 10 strains of Aerobacter aerogenes produced mucoid colonies when cultured at 35°C, although after 7 days at room temperature mucoidness was present in all except one. This is surprising in view of the fact that mucoid strains of Aerobacter generally show this characteristic after incubation at 35°C to 37°C. The examination of India-ink preparations revealed the possession of capsules and presence of slime by all except the non-mucoid strain; but slime was not generally present in very large amounts and in three cases was only slight.

(2) In contrast to the aerogenes strains, of 14 typical Aerobacter cloacae and 2 which were atypical in being

(i) non-motile and (ii) gas-forming in glycerol medium 13 produced mucoid colonies at 35°C, although with 4 of them the mucoidness was only slight. All of them were mucoid after 5 days at room temperature and in all cases they were purely slime-forming strains. Even the non-motile strain was non-capsulated. On the other hand, 5 out of 6 additional strains of cloacae which were atypical in their inability to liquefy gelatine, failed to produce mucoid growth at 35°C, although all but one of them did so after 7 days at room temperature. As with the typical strains, none of them were capsulated and all were abundant in slime-formation. It will be shown later that the majority of these non-gelatine liquefying strains are also atypical in being late in fermenting "sugar" media. It is thought that the failure to produce mucoidness at 35°C, as do the typical strains of cloacae and the reduction in biochemical activity may have resulted from the treatment of heat and cold, to which the organisms were subjected during the preparation of ice cream, and that this may also apply to the strains of aerogenes which failed to be mucoid when grown at 35°C and are shown later to have been atypical to their inability to produce gas in inositol.

3. Intermediate Group.

Additional tests, carried out on the 24 intermediate strains, revealed that two of them produced indole when grown in peptone water for 24 hours. They therefore correspond to Wilson's intermediate type II. The remaining 22 were all indole negative. All strains failed to produce gas in lactose broth when grown at 44°C, i.e. were Eijkman negative. Two strains, liquefied gelatine after 4 months and therefore correspond to the irregular IV, intermediate-like strain of Wilson. Thus the 24 intermediate strains may be sub-divided on the basis of the above additional tests into:

Type I :- 20 strains.

Type II:- 2 strains

Irregular IV, intermediate-like:- 2 strains.

Sugar-Fermentation-Reactions.

The sugar-fermentation reactions of all strains of coliform bacilli isolated from ice cream were observed, the following "sugars" being employed :- Glucose, lactose, sucrose, dulcitol, mannitol, glycerol

and inositol. The fermentable substances were incorporated in peptone water in the proportion of 0.5%, and neutral red was added as an indicator.

The results are shown in Tables XVII., XVIII. and XIX.

1. Twenty-three strains of Aerobacter-aerogenes isolated from ice cream were tested for their fermentation reactions in "sugar" media. Eighteen of them were in other respects typical Aerobacter aerogenes type I, 3 were type II and 2 were atypical strains, viz:- (a) type I, but motile and slow to grow in citrate medium and (b) type II, capable of liquefying gelatine. The results are tabulated in Table XVII.

All strains, both typical and atypical fermented glucose, lactose and mannitol with acid and gas production. Sucrose was fermented by every strain except one which was an atypical type I ; see (a) above. Dulcitol was fermented by only four strains, one of which was the atypical, gelatine-liquefying strain. Glycerol was fermented with acid and gas production by all except one strain which produced acid only. The results with inositol were variable:- Of the 18 type I strains, 2 produced acid and a small bubble of gas after three days incubation, 9 produced acid and no visible gas, 6 produced a very slight or late acid

TABLE XVII.

Fermentation Reactions of Aerobacter aerogenes.	Ref.No.	Glucose	Lactose	Sucrose	Dulcitol	Mannitol	Glycerol	Inositol
Typical Strains	83264 (1)	AG	AG	AG	—	AG	AG	Sl.A
	" (2)	AG	AG	AG	—	AG	AG	Sl.A
	83265 (1)	AG	AG	AG	AG	AG	AG	A
	83889 (1)	AG	AG	AG	—	AG	AG	A
	84326 (1)	AG	AG	AG	—	AG	AG	A
	" (2)	AG	AG	AG	—	AG	AG	A
	" (3)	AG	AG	AG	—	AG	AG	A
	" /2 (1)	AG	AG	AG	—	AG	AG	A
Type I.	" /2 (2)	AG	AG	AG	—	AG	AG	A
	84392/2 (1)	AG	AG	AG	—	AG	AG	A
	84434 (1)	AG	AG	AG	—	AG	AG	-
	" (3a)	AG	AG	AG	—	AG	AG	Sl.A
	" (3b)	AG	AG	AG	—	AG	AG	Sl.A
	84438 (3a)	AG	AG	AG	—	AG	AG	Sl.AG
	85903	AG	AG	AG	AG	AG	AG	Sl.AG
	I.C. 2 (1)	AG	AG	AG	—	AG	AG	Sl.A
	" (2)	AG	AG	AG	AG	AG	AG	A
	W (2)	AG	AG	AG	—	AG	A	Sl.A
Type II.	87376 /2(1)	AG	AG	AG	—	AG	AG	AG
	" (2)	AG	AG	AG	—	AG	AG	AG
	" (3)	AG	AG	AG	—	AG	AG	AG
Atypical strains Motile and slow Citrate-utiliser Type I.	83921 (2)	AG	AG	—	—	AG	AG	-
Gelatine Liquefying Strain Type II.	84034/2 (1)	AG	AG	AG	AG	AG	AG	AG

AG = Acid and gas Sl.AG = late or weak reactions

A = Acid Sl.A = " " " "

reaction and 1 had no effect on the sugar. The 3 strains of type II Aerobacter aerogenes, on the other hand produced acid and gas in large amounts. Of the two atypical strains, the motile one failed to produce any reaction in inositol, but the gelatine-liquefying strain produced acid and gas in that and all the other "sugars" tested.

The variability of the fermentation reactions in inositol by strains of Aerobacter aerogenes and the absence of visible gas production by the majority is surprising in view of the fact that according to Mackie quoted by Malcolm (1935) and (1938), the ability to ferment inositol with acid and gas production is characteristic of Aerobacter aerogenes and affords a means of differentiating this species from other coliform bacilli.

Confirmation of the writer's results of the reactions in inositol-peptone water was obtained from Dr. James Duguid, who grew the irregular strains with Aerobacter aerogenes obtained from other sources. These latter produced acid and gas, whereas the strains from ice cream gave the reactions already recorded.

2. Thirty-four strains of Aerobacter cloacae were tested for their fermentation reactions when grown in "sugar" media. Twenty-six were typical in other

TABLE XVIII.

Fermentation Reactions of Aerobacter cloacae	Ref. No.	Glucose	Lactose	Sucrose	Dulcitol	Mannitol	Glycerol	Inositol.
Typical strains .	82602 (1)	AG	AG	AG	-	AG	A	A
	" (2)	AG	AG	AG	-	AG	A	A
	82636 (2)	AG	AG	AG	-	AG	A	A
	83259 (1)	AG	AG	AG	-	AG	A	-
	83919 (1)	AG	AG	AG	-	AG	A	A
	" (2)	AG	AG	AG	-	AG	A	A
	83923 (1)	AG	AG	AG	-	AG	A	A
	" (2b)	AG	AG	AG	-	AG	A	-
	" (3)	AG	AG	AG	-	AG	A	-
	83989	AG	AG	AG	-	AG	A	A
	84034 (1)	AG	AG	AG	-	AG	A	A
	" (3)	AG	AG	AG	-	AG	A	A
	" /2(2)	AG	AG	AG	-	AG	A	A
	84035 (1)	AG	AG	AG	-	AG	A	A
	" /2(2)	AG	AG	AG	-	AG	A	A
	84100	AG	AG	AG	-	AG	A	SLA
	84434 (2)	AG	AG	AG	-	AG	A	A
	84438 (1a)	AG	AG	AG	-	AG	A	SLA
	" (1b)	AG	SLAG	AG	AG	AG	A	A
	" (2)	AG	AG	AG	-	AG	A	SLA
	84793 (1)	AG	AG	AG	-	AG	A	SLA
	" (2)	AG	AG	AG	-	AG	A	SLA
	" (3a)	AG	AG	AG	-	AG	A	SLA
	87265 (1)	AG	AG	AG	-	AG	A	A
	87377/2(1)	AG	AG	AG	-	AG	A	A
	82637 (2)	AG	AG	AG	-	AG	AG	A
Atypical strains								
Non-gelatine liquefying strains.	82561 (1)	AG	SLAG	AG	-	AG	A	-
	82639 (2)	SLAG	SLAG	AG	-	SLAG	A	-
	83889 (3)	SLAG	SLAG	SLAG	-	SLAG	SLA	-
	83923 (2a)	SLAG	SLAG	SLAG	-	SLAG	SLA	-
	83924 (1)	AG	AG	AG	-	AG	A	-
	84035/2(3)	AG	AG	AG	-	AG	A	-
Non-motile strains	84439 (1)	AG	AG	AG	-	AG	A	A
	" (2)	AG	AG	AG	-	AG	A	A

AG = acid and gas SLAG = late or weak reactions.
A = acid SLA = " " "

respects including their positive motility and their ability to liquefy gelatine. Six were atypical in failing to liquefy gelatine and 2 in being non-motile.

The "sugars" tested were glucose, lactose, sucrose, dulcitol, mannitol, glycerol and inositol.

The results of the tests are tabulated in Table XVIII. The reaction of the 26 typical strains are summarised as follows:

Glucose:- Acid and gas - 26 strains

Lactose:- Acid and gas - 26 strains

Sucrose:- Acid and gas - 26 strains

Dulcitol:- Negative - 25 strains;
Acid and gas - 1 strain

Mannitol:- Acid and gas - 26 strains

Glycerol:- Acid - 25 strains;
Acid and gas - 1 strain

Inositol:- Acid - 17 strains
Slight or late acid - 6 strains -
negative - 3 strains.

Of the 6 non-gelatine-liquefiers, 4 were slow in fermenting one or all of the sugars although the end results of their reactions were eventually the same as those of the typical strains, except for inositol from which no acid was produced by any of these six atypical strains.

The two non-motile strains gave reactions which were similar to those of the typical strains, inositol and glycerol being fermented by both with production of acid but without visible gas-formation. Thus the loss of ability to liquefy gelatine appears to be correlated with a certain degree of reduction in fermentative powers, noticeable in the slow-production of visible gas from the "sugars" and complete loss of ability to produce acid from inositol.

The loss of motility, on the other hand, does not appear to be associated with any change in fermentative ability as far as can be seen from the two examples studied.

3. The "sugar" fermentation reactions of 7 strains of Escherichia coli are shownin Table XIX. All fermented glucose, lactose, and mannitol with acid and gas production, 2 failed to ferment sucrose and these were also t he only ones to bring about fermentation of dulcitol. Two produced acid and gas in glycerol-medium and 5, acid without gas production. With inositol, the reaction was negative in every case, or resulted in a very slight acid reaction.

4. No distinction could be drawn between Intermediate strains types I and II and Irregular type IV on the

TABLE XIX.

Fermentation Reactions of Escherichia coli. and Intermediate strains.		Glucose	Lactose	Sucrose	Dulcitol	Mannitol	Glycerol	Inositol.
Escherichia Coli.	82602 (2E)	AG	AG	AG	—	AG	AG	Sl.A
	83919 (3)	AG	AG	AG	—	AG	AG	Sl.A
	83919 (1E)	AG	AG	AG	—	AG	A	Sl.A
	83919 (3E)	AG	AG	AG	—	AG	AG	Sl.A
	84034/2 (3)	AG	AG	AG	—	AG	A	Sl.A
	84034/2(1E)	AG	AG	AG	—	AG	A	—
	87073 (2E)	AG	AG	--	AG	AG	A	Sl.A
	87073 (3E)	AG	AG	--	AG	AG	A	Sl.A
Intermediate Strains								
Type I.	82557 (2)	AG	AG	AG	—	AG	AG	Sl.A
	82558 (2)	AG	AG	A	AG	AG	AG	—
	82559 (2a)	AG	AG	AG	AG	AG	AG	AG
	82559 (2b)	AG	AG	AG	AG	AG	AG	A
	82563 (2)	AG	AG	AG	AG	AG	AG	Sl.A
	82636 (1)	AG	AG	AG	—	AG	AG	—
	83261 (2)	AG	Sl.A	—	—	AG	A	A
	83265 (2)	AG	AG	AG	—	AG	AG	A
	83258 (2)	AG	AG	AG	AG	AG	AG	Sl.A
	83888 (1)	AG	AG	AG	—	AG	AG	A.
	83920 (1)	AG	AG	AG	AG	AG	AG	Sl.A
	83921 (1)	AG	AG	AG	—	AG	AG	A.
	83970 (1)	AG	AG	AG	—	AG	AG	A
	83970 (2)	AG	AG	AG	—	AG	AG	A
	83970 (3)	AG	AG	AG	—	AG	AG	SL.AG
	84034 (2)	AG	AG	AG	SL.AG	AG	AG	—
	87263 (1)	AG	AG	AG	—	AG	A	Sl.A
	87263 (2)	AG	AG	AG	—	AG	A	Sl.A
	83921 (3)	AG	AG	AG	—	AG	AG	A
	82603 (2)	A	A	—	—	A	A	A
Type II.	82561 (2)	AG	AG	AG	AG	AG	AG	AG
	82635 (2)	AG	AG	AG	—	AG	AG	AG
Irregular IV.	82559 (1)	AG	AG	AG	—	AG	AG	A
	83890 (2)	AG	AG	AG	—	AG	A	—

AG = Acid and Gas Sl.AG = late or weak reactions.

A = Acid Sl.A = " " "

bases of their fermentation reactions. (See Table XIX.). Among the 20 type I strains, was one which produced acid without visible gas formation in all "sugars" except sucrose and dulcitol which were unaffected,and one which produced acid only in lactose-medium. Apart from these two, all strains,including the two type II strains and the irregulars,fermented glucose, lactose and mannitol with acid and gas production. Sucrose was fermented 21 out of 24 times, the three exceptions all belonging to type I. Acid and gas was formed from glycerol by 16 of the 20 type I strains, by both the type II and by one of the irregulars and acid without gas,by 4 type I and by the second of the irregular strains. The reactions in inositol medium were even more variable, acid and gas being formed by one of the type I and one of the type II strains, and acid and late gas production by another of the type I strains. Acid without visible gas was produced by 10 strains, including a type II and an irregular and a late or slight acid reaction by 6 strains, all of type I. Five strains including an irregular, had no visible effect on the sugar.

TABLE XX.

Comparison of Fermentation Reactions of all species of Coliform Bacilli.

		Glucose	Lactose	Sucrose	Dulcitol	Mannitol	Glycerol	Inositol
Aerobacter aerogenes	AG	23	23	22	4	23	22	4 + 2 weak reactions
	A	0	0	0	0	0	1	9 + 6 weak reactions
	-	0	0	1	19	0	0	2
Aerobacter cloacae	AG	26	26	26	1	26	1	0
	A	0	0	0	0	0	25	17 + 6 weak reactions
	-	0	0	0	25	0	0	3
Escherichia coli	AG	7	7	5	2	7	2	0
	A	0	0	0	0	0	5	6 weak reactions
	-	0	0	2	5	0	0	1
Intermediate strains	AG	23	22	21	8	23	19	2 + 1 weak reaction
	A	1	2	0	0	1	5	10 + 6 weak reactions
	-	0	0	3	16	0	0	5

AG = Acid and gas
A = Acid
- = No reaction.

The numbers refer to the number of strains producing or not producing a reaction.

Conclusions:-

The results of the "sugar" fermentation reactions of all strains of coliform bacilli examined are summarised in Table XX. It is seen that no information of any differential value is contained from the reactions in glucose, lactose or mannitol, from which acid and gas were produced in practically every case; nor are the reactions in sucrose specific for any one group, since acid and gas were produced by all except 6 strains, some of which occurred in each of the three groups, Escherichia, Aerobacter and Intermediate. Little is gained from the inability of most strains to ferment dulcitol, positive reactions being produced occasionally by members of all groups. The fermentation of glycerol with acid and gas production has already been shown to be of value in differentiating Aerobacter aerogenes from Aerobacter cloacae which fails to produce visible gas from this "sugar". Escherichia coli and intermediate strains may or may not produce acid and gas from that "sugar".

Inositol fermentation by strains of coliform bacilli isolated from ice cream is so variable as to be of no practical value for differential purposes, although it was found that in no case was gas produced by

strains of cloacae, or coli. In view of the fact, however, that acid and gas production is generally considered to be a valuable criterion in determining species of Aerobacter aerogenes, the loss of the ability to produce visible gas from it, by so many of the strains and the weak acid reaction produced by them, suggests that physiological changes may have taken place in the bacteria as a result of either the heat-treatment, or if introduced into the ice cream mix after heat-treatment, then to the severe cooling and freezing to which they were submitted during the manufacture of the ice cream.

Summary of Examination of Coliform Bacilli isolated from Ice Cream.

1. Ninety strains of lactose-fermenting organisms were isolated from 53 samples of ice cream which had shown a positive reaction to the presumptive test for coliform bacilli.

By means of the methyl-red, Voges-Proskauer and citrate-utilisation tests, the strains were divided into three grous, viz:-

1.	Escherichia	:-	9 strains.
2.	Aerobacter	:-	57 "
3.	Intermediate	:-	24 "
	Total		90

2. The 9 Escherichia strains were found to be Eijkman positive and therefore correspond to Escherichia coli, Bergey. They were also indole, positive and similar to Bact. coli type I Wilson. Although Bergey does not discriminate between indole positive and indole negative strains, it is proposed here to refer to those which produce indole as Escherichia coli type I and those which are similar but fail to produce indole as Escherichia coli type II. All the strains isolated from ice cream were found to be of type I.

3. On the basis of gelatine-liquefaction, glycerol-

fermentation and motility tests, the Aerobacter group was subdivided into 2 species thus:-

	Gelatine-liquefaction	Glycerol-fermentation	Motility	No. of strains
1. Aerobacter aerogenes	-	acid and gas	-	21
2. Aerobacter cloacae	+	acid	+	26
	Irregular strains			10
			Total	57

If the results of two out of the three tests were used to identify an organism, the result of the third test failing to fit into the scheme, then the 10 irregular Aerobacter strains could be identified as follows:

	Irregular in being:-	No. of strains
Aerobacter aerogenes	motile	1
" "	gelatine-liquefying	1
Aerobacter cloacae	non-motile	2
" "	non-gelatine-liquefying	6
	Total	10

4. Indole was produced by 2 of the typical strains of Aerobacter aerogenes and by the irregular gelatine-liquefying strain which thereby corresponds to

B. oxytocus. Malcolm. According to Wilson's classification these three indole producers are designated type II aerogenes.

Similarly 2 of the intermediate strains produced indole and are therefore type II intermediates whereas the non-indole producers are type I. Two of these latter strains proved to be gelatine-liquefiers and are classed as irregular type IV by Wilson's method.

5. Mucoid-colonies were produced at 35°C by only 4 of 12 strains of Aerobacter aerogenes and by 11 of the 12 when grown at room temperature. 14 of the 22 strains of Aerobacter cloacae produced mucoid colonies at 35°C and all except one of them, at room temperature. Of the 8 cloacae strains which failed to produce mucoid colonies at 35°C, 5 were irregular in being non-gelatine liquefiers.

Capsules were produced by all except one of the aerogenes strains, the exception being irregular also in its motility and slow-utilisation of citrate. This was in contrast to the absence of capsules by all strains of Aerobacter cloacae both regular and irregular. This confirms the identification of the irregular strains on the basis of the results of two out of three tests (vide paragraph 3).

Slime was produced by all Aerobacter strains except the two non-mucoid ones, but more of it was produced by cloacae than by aerogenes.

6. No value was obtained for differential purposes from the results of the fermentation reactions in glucose, lactose, sucrose, dulcitol or mannitol peptone water.

As shown in paragraph 3, the production of acid and gas in glycerol-peptone water was diagnostic for Aerobacter aerogenes whereas acid production alone was characteristic of cloacae. Only one aerogenes strain failed to produce gas as well as acid amd only one cloacae produced gas with acid.

Fermentation of inositol by Aerobacter aerogenes was found to be very variable and unlike the usual reaction from this species, the strains isolated from ice cream failed to produce gas, with the exception of the type II strains and 2 others which produced only a weak reaction.

7. It was found that of 6 non-gelatine-liquefying strains of cloacae, 4 were slow sugar fermenters and 5 did not produce mucoid colonies at 35°C as did the other strains of cloacae.

Since the majority of Aerobacter aerogenes strains failed to give a typical acid and gas reaction

in inositol-peptone water and failed to produce mucoid colonies at 35°C as is usual with this species, it is possible that the heating and cooling to which the organisms are submitted in the ice cream mix may bring about biological changes in the organisms which result in the abnormal features of these cloacae and aerogenes strains.

8. By using the above scheme of classification and by omitting those strains which proved to be duplicates from the same sample the coliform organisms isolated from ice cream were identified as follows:-

Escherichia coli type I	-	5 strains
Aerobacter aerogenes type I	-	13 "
" " type II	-	2 "
Aerobacter cloacae	-	21 "
Intermediate type I	-	16 "
" type II	-	2 "
" (irregular type IV Wilson)	-	2 "
	Total	61

9. The relative proportions of the various types of coliform bacilli correspond closely with those of Bardsley (1934) and (1938) who reported that the predominating type of coliform bacilli in ice cream was

B. lactis aerogenes (Bact. aerogenes, Wilson) which accounted for 61% of the strains in 1934 and 45.8% in 1938. Since Bardsley failed to differentiate aerogenes from cloacae, her figures may be compared with the total number of aerobacter strains obtained in the present investigation, viz., 59%, a figure which is not dissimilar to her findings.

It has been shown here that Aerobacter cloacae is more commonly present in ice cream than Aerobacter aerogenes, and accounted for 58% of the total Aerobacter strains.

Intermediate and irregular strains formed 32.7% of the total, a figure which corresponds closely to Bardsley's 35.8% for 1938.

Escherichia coli strains accounted for 8% of the total (c.f. Bardsley's percentages which were 7% for 1934 and 13.9% 1938).

Appendix to SECTION II., Sub-section 2.

The Possibility of Variants of Aerobacter aerogenes arising as a result of heat-treatment and freezing of Ice Cream "Mix".

INTRODUCTION:-

Owing to the fact that the majority of Aerobacter aerogenes strains isolated from ice cream were atypical in a number of respects, the chief being their slowness to produce mucoid colonies at 35°C, it was thought that these organisms might be variants of typical strains, resulting from the effects of the heat-treatment and freezing to which they were submitted in the ice cream "mix."

In order to test this possibility, pure cultures of typical Aerobacter aerogenes from another source were added to tubes of melted ice cream and submitted to heating, cooling and freezing in the laboratory. After treatment they were plated out on MacConkey agar plates in order to determine whether all the organisms which survived were still able to produce mucoid colonies within 24 hours at 35°C or whether non-mucoid variants had been formed. It was decided that any non-mucoid colonies which might arise should be sub-cultured and their biological character determined.

A preliminary experiment was carried out as follows :-

Experimental:

1. A peptone water culture of Aerobacter aerogenes was prepared, using 0.05% peptone water which is known to be just sufficient to permit the growth of approximately 100,000,000 organisms in 18-24 hours at 35°C.

10 ml. amounts of a sample of ice cream, tested and found to be free from coliform organisms, were added to each of 5 test tubes containing 1 ml. of the peptone water culture of Aerobacter aerogenes.

The tubes and contents were then treated as follows:-

(1) By heating in a water bath at 65.5°C for 15 minutes and 30 minutes.

(2) By heating in a water bath at 65.5°C for 15 minutes and 30 minutes and then afterwards cooling by immersing in an ice-salt mixture for 30 minutes and refrigerating for 24 hours at less than 0°C.

(3) By cooling and refrigerating only, without previous heat-treatment.

After treatment 2 plates of MacConkey agar were inoculated with the contents of each of the tubes and were then incubated at 35°C and examined after 24 hours for mucoid and non-mucoid colonies.

The results are shown in the following Table :-

Ice Cream + Aerobacter aerogenes Tube	Growth on MacConkey Plates inoculated immediately after treatment.
(1) Heated for 15 minutes	No growth
(2) Heated for 15 minutes and frozen	No growth
(3) Heated for 30 minutes	No growth
(4) Heated for 30 minutes and frozen	No growth
(5) Frozen only	Mucoid growth
(6) Untreated	Mucoid growth
(7) Ice cream control	No growth

Conclusions:- A pasteurisation temperature of 65.5°C (150°F) (the minimum temperature recommended in the Ice Cream Regulations) was sufficient to prevent the growth of the test strain of typical Aerobacter aerogenes on MacConkey agar when applied for 15 minutes. As a result of this, there was no growth on any of the MacConkey plates inoculated with the heat-treated ice cream containing Aerobacter. The untreated mixture and the mixture which was submitted to freezing and refrigeration resulted in a mucoid growth only on the MacConkey plates.

2. The experiment was repeated with sub-lethal doses of heat-treatment, viz. 55°C. for periods of 5, 10, 15 and 30 minutes. After heat-treatment the contents of the tubes were plated out on MacConkey agar before and after

immediate freezing and refrigerating as in the previous experiment. In order to detect any possible variants which might be unable to produce colonies within 24 hours, the tubes and contents were held at laboratory temperature for 24 hours and the contents again plated out. All inoculated MacConkey plates were incubated at 35°C for 24 hours and afterwards held at laboratory temperature for a week.

The results were as follows:-

Ice Cream + Aerobacter aerogenes Tube	Growth on MacConkey plates at 35°C inoculated with culture immediately After treatment.	after 24 hours at laboratory temperature.
(1) Heated 55°C for 5 minutes	Mucoid	Mucoid
(2) Heated 55°C for 5 minutes and frozen	Mucoid	Mucoid
(3) Heated 55°C for 10 minutes	Mucoid	Mucoid
(4) Heated 55°C for 10 minutes and frozen	Mucoid	Mucoid
(5) Heated 55°C for 15 minutes	Mucoid	Mucoid
(6) Heated 55°C for 15 minutes and frozen	Mucoid	Mucoid
(7) Heated 55°C for 30 minutes	No growth	No growth
(8) Heated 55°C for 30 minutes and frozen	No growth	No growth
(9) Frozen only (including refrigeration for 24 hours)	Mucoid	Mucoid
(10) Untreated	Mucoid	Mucoid
(11) Ice cream control (uninoculated)	No growth	No growth

Conclusions:-

30 minutes at 55°C was sufficient to inhibit the growth of Aerobacter aerogenes on MacConkey agar plates inoculated with the mixture immediately after heat-treatment and again after standing at laboratory temperature for 24 hours.

The ice cream containing Aerobacter heated for periods of time up to 15 minutes at 55°C with and without subsequent freezing, resulted in the growth of mucoid colonies only and no non-mucoid variants were observed even after the mixtures had stood at laboratory temperature for 24 hours before plating and the plates for 1 week after inoculation.

3. Finally, the experiment was repeated with another make of ice cream which was heated in a boiling-water bath for 1 hour to destroy any coliform organisms present. To 4 tubes each containing 10 ml. of the melted sterile ice cream, was added 1 ml. of a suspension of Aerobacter aerogenes in saline (100,000,000 per ml.). The tubes and contents were then treated as before viz.:- Tube (1), heated at 55°C for 15 minutes; tube (2), heated as (1) and then frozen by immersing in an ice-salt mixture for 30 minutes and refrigerated for 24 hours at less than 0°C; tube (3), frozen and refrigerated only. Tube (4) was untreated. MacConkey agar

plates were inoculated with a loopful of the contents of each of the 4 tubes, immediately after the treatment and again after they had stood at atmospheric temperature for 24 hours.

As in the previous experiments, only mucoid colonies appeared on the plates both on those inoculated with the mixtures immediately after treatment and after standing for 24 hours at laboratory temperature. Non-mucoid colonies failed to appear even after the plates had stood at laboratory temperature for several days.

Summary:-

(1) An attempt was made to produce non-mucoid variants of a typical strain of Aerobacter aerogenes by adding suspensions of the organisms to ice cream and then submitting the mixtures to sub-lethal heat-treatment, freezing and refrigeration.

No non-mucoid variants resulted in any of the three experiments carried out.

(2) Temperatures of 65.5°C for 15 minutes and 55°C for 30 minutes were sufficient to inhibit the growth on MacConkay agar of the test strain of Aerobacter aerogenes in ice cream.

SECTION III.

Being further investigations into certain factors which affect the results of the Plate Count and Methylene Blue Reduction Tests as applied to Ice Cream.

Sub-section 1. The effect of Heat and Cold on the Bacterial Flora of Ice Cream.

Sub-section 2. Methylene Blue Reduction by
(a) Aerobic Spore-forming Bacilli.
(b) Other Organisms isolated from Ice Cream.

Sub-section 1: The Effect of Heat and Cold on the Bacterial Flora of Ice Cream.

INTRODUCTION:

It has been suggested in Section I. that the plate counts of samples of ice cream, tested immediately after being in a frozen condition, may not give a fair indication of the number of viable bateria present, since many of them may be in a "dormant" or "attenuated" state as a result of the heating and cooling processes.

It was shown by Eijkman (1908) that sub-lethal heating may cause sporing organisms to become "dormant" and to remain in that state for long periods. Burke et al (1925) described how vegetative cells of non-sporing bacilli may remain "dormant" for a time after the initial "lag" phase shown by organisms when first transferred to a fresh medium. Allen (1923) investigating the effect of pasteurisation on a number of organisms isolated from milk, showed that, while some species are killed outright by pasteurisation, others, with thermal death points a little above pasteurisation temperatures may be merely "attenuated", i.e. one or more of their physiological functions may be altered,and this is often manifested in a decreased rate of growth and multiplication.

Others again, whose thermal death points are well above the temperature of pasteurisation are either unaffected or they may be even stimulated to more active growth by the treatment. Bushnell, quoted by Allen suggests that rapid cooling is an "attenuating" influence in itself, causing devitalisation of the organisms.

A number of workers have shown that as a result of its high sugar content, many of the organisms in ice cream withstand temperatures which would be lethal to them in milk or water. (See Beavens (1930); Anzulovic (1932); Fay (1934)). Fay makes the suggestion that the sugar prevents coagulation of cell colloids and thereby enables the organisms to withstand greater temperatures than normally. Bancroft and Richter (1931) state that cell colloids may at first be reversibly coagulated by heat and at this stage the organism will become abnormal in character, but able to regain its normal functions when transferred to a favourable medium. With increased heating, the cell protein becomes less and less reversibly coagulated and finally irreversibly so resulting in the death of the organisms.

The bacterial flora of heat-treated ice cream

may therefore consist of (a) thermoduric organisms, unaffected by the heat-treatment; (b) aerobic spore-forming bacilli in a dormant state as a result of the heat-treatment; (c) organisms "attenuated" in their ability to grow or multiply as a result of the heat-treatment (d) organisms which have contaminated the product after heat-treatment, some of which are devitalised as a result of the sudden cooling and freezing, to which they were subjected during the preparation of the ice cream.

Fay showed that by adding sucrose to the agar medium, higher plate counts of the survivors of heat-treatment resulted, and he suggested that cells, damaged but not destroyed by heat, require the additional nourishment provided by the carbohydrate, to enable their normal vitality to be restored.

It may be that by holding ice cream samples at atmospheric temperature (or at a controlled temperature of 20°C) for a time, before carrying out the plate count test, the same result may be achieved, since any organisms which are in a dormant or "attenuated" condition may be better able to recover their vitality in the presence of the additional nutriments, including sugar, present in the ice cream.

The following experiments were carried out in order to determine the effect of heat and cold on two species of bacteria isolated from ice cream, viz., Bacillus cereus and Aerobacter aerogenes and to ascertain to what extent the organisms are killed, and to what extent merely "attenuated," so that they remain dormant for a period and are undetected by the plate count tests carried out immediately after being in a frozen condition.

EXPERIMENTAL.

A. The Effect of Heat and Cold on Cultures of Bacillus cereus.

Experiment I. shows the effect on the plate count of first heating and then rapidly cooling and freezing bacterial cultures.

The test organism was B. cereus grown in sterile whole milk for 24 hours and then diluted by adding 1 ml. of the culture to 100 ml. sterile milk. After shaking vigorously 100 times, the mixture was divided into four portions, and plate counts carried out on each of them as follows:

Portion 1. Immediately.

Portion 2. After heating at 70°C (158°F) for 15 minutes.

Portion 3. After heating at 70°C for 15 minutes and then cooling rapidly by immersing in an ice and salt mixture and refrigerating at -6°C for 17 hours.

" 4. After cooling rapidly and freezing at -6°C for 17 hours without first heating.

After the initial plating, all portions were held at 20oC for 17 hours and the plate count test again carried out.

All tests were done in duplicate; one plate being incubated at 37°C for 48 hours and one at 22°C for 3 days. Results are given in Table XXI.

It is seen that heating alone reduced the number of bacteria represented by the plate count from 269,000 to 1,550. Heating and cooling reduced the number to 1,100 and cooling alone reduced the number to 35,000.

Testing each of these portions a second time after they had been allowed to stand at 20°C for 17 hours give interesting results. The heated portion and the heated and frozen portion showed a rise in count from 1,000 approximately to 110,000 and 200,000 organisms per ml. respectively. The portion which was cooled and frozen without first heating showed a rise

TABLE XXI.

Effect of heating and rapid cooling and refrigeration on a milk culture of B. cereus diluted 1 in 100.

B. cereus culture	Plate Count (orgs/ml. tested.) immediately		after 17 hours at 20°C	
	37°C	20°C	37°C	20°C
1. Untreated	260,000	250,000	1,500,000	1,200,000
2. Heated	1,550	1,540	110,000	110,000
3. Heated. cooled and refrigerated	1,100	900	200,000	140,000
4. Cooled and refrigerated	35,000	35,000	6,300,000	6,200,000

of from 35,000 to over 6,000,000, a figure greater than the plate count of the original diluted culture after it had been held at + 20°C for 17 hours. The reduction in the count after freezing from 260,000 to 35,000 does not appear to represent the number of organisms killed, but rather those which were rendered "dormant" by the process and which became revitalised and multiplied during the subsequent period at + 20°C.

Experiment 2 shows the effect of cooling and freezing without previous heat-treatment on cultures of Bacillus cereus.

The organism was grown in nutrient broth for 24 hours at 37°C. The culture was diluted by adding 1 ml. to 100 ml. sterile broth, which was vigorously shaken 100 times. The diluted culture was then divided into two portions and plate counts carried out on each of them as follows:

Portion 1. Immediately.

" 2. After cooling rapidly by immersing in an ice-salt mixture and then refrigerating at - 6°C for 17 hours.

Both portions were then held at 20°C for 17 hours and the plate count test repeated on each.

In preparing the dilutions for the test, 3 screw-capped bottles containing 9 ml. ½ strength Ringer solution were used and after the addition of the culture, each bottle was vigorously shaken one hundred times to break up any clumps of bacteria.

Results: The results of the tests are shown in Table XXII from which it is seen that the bacterial count of the untreated broth culture, as measured by the plate count was reduced from 400,000 organisms per ml. to 200, by the sudden cooling and refrigeration at - 6°C for 17 hours. By holding this sample afterwards at + 20°C for 17 hours, the plate count rose to 90,000 per ml. The reduction in the number of organisms from 400,000 per ml. to 200 is thought to be due to attenuation of many of them rather than to actual killing, and the subsequent rise from 200 to 90,000 which follows the period of holding at + 20°C for 17 hours, may indicate renewed vitality of the "attenuated" organisms rather than multiplication of only the 200 unaffected ones.

Experiment 3: This was similar in outline to Experiment 2 except that the organism B. cereus was grown in sterile milk for 24 hours and diluted by adding 0.5 ml. to 100 ml. sterile milk. Plate counts were carried out before

TABLE XXII.

Effect of rapid cooling and refrigeration on a broth culture of *B. cereus*, diluted 1 in 100.

B. cereus culture	Plate count (orgs./ml.) Tested	
	immediately	after 17 hours at 20°C.
1. Untreated	400,000	Uncountable
2. Cooled and refrigerated	200	90,000

TABLE XXIII.

Effect of rapid cooling and refrigeration of a culture of *B. cereus* in milk, diluted 1 in 200

B. cereus culture	Plate Count(orgs/ml.) tested			
	immediately		after 17 hr. at 20°C	
	37°C	20°C	37°C	20°C
1. Untreated	220,000	220,000	460,000	500,000
2. Cooled and Refrigerated	12,000	4,000	7,000	4,000

and after rapid cooling and freezing and again after immersion in a 20°C water bath for 17 hours. The plates were duplicated, one set incubated at 37°C and the other at 22°C. Results are given in Table XXIII. In this case cooling and freezing resulted in a drop from 220,000 organisms per ml. to 12,000. There was however no increase in the count after a period of 17 hours at 20°C. Either the organisms were killed by the severe chilling which is unlikely or else they continued in the dormant state for longer than 17 hours at + 20°C. There was no significant difference between the plate count at 37°C and that at 22°C and continued incubation failed to produce additional colonies on the agar.

B. The Effect of Heat and Cold on Aerobacter aerogenes in Ice Cream.

In order to observe the effect of heat and cold on non-spore-forming bacilli, an experiment similar to the last was carried out with a culture of Aerobacter aerogenes.

Experimental:

One ml. quantities of a 24 hour peptone water culture of Aerobacter aerogenes were added to each of 4 tubes containing 10 ml. amounts of sterile ice cream.

The contents of the four tubes were then treated as follows:-

1. Untreated.
2. Heated at 55°C for 15 minutes.
3. Heated at 55°C for 15 minutes, and immediately cooled by immersing in an ice and salt mixture for 30 minutes before refrigerating overnight at less than 0°C.
4. Cooling as above, and refrigerating overnight without previous heat-treatment.

A plate count test was carried out on the contents of each tube, immediately after completion of the treatment, and again after standing for 24 hours at atmospheric temperature. The results of these tests are shown in Table XXIV.

Results.

It is seen that heat-treatment alone, reduced the number of organisms, represented by the plate count test, from 4,000,000 to 80,000 per ml. Freezing, following heat-treatment, reduced the plate count still further to 36,000 per ml., while freezing without previous heating, had the effect of reducing the count to 420,000 per ml.

TABLE XXIV.

Effect of heating and then rapid freezing and refrigeration on a suspension of *Aerobacter aerogenes* in Ice Cream

	Suspension in ice cream	Plate Count (orgs.per ml.) immediately	after 24 hours
1.	Untreated	4,000,000	144,000,000
2.	Heated	80,000	20,000
3.	Heated, frozen and refrigerated	36,000	4,000
4.	Frozen and refrigerated	420,000	120,000,000

On testing a second time, after the tubes had stood at atmospheric temperature for 24 hours, plate counts resulted which, in the cases of the untreated portion and the one which had been frozen without heating, showed a marked increase on the previous result. The plate counts of both portions which had been heated, showed no increase but instead, a decrease resulted in each case.

These results suggest that the original plate counts of both heat-treated portions, represented the total number of survivors; with the frozen portion on the other hand, the increase in the plate count after 24 hours, to almost the same amount as the untreated portion, suggests that, in addition to the survival of 420,000 organisms, revealed by the first test, there were many others which were in a "dormant" or "attenuated" condition, and which regained their normal vitality during the pre-testing period.

Summary and Conclusions.

1. The results of the experiments with B. cereus and Aerobacter aerogenes as test organisms, show that not only did heating followed by cooling and refrigeration reduce the plate counts of the cultures, but sudden cooling and refrigeration without previous heat-treatment, also brought about a considerable reduction in numbers.

2. When the B. cereus cultures were tested a second time, after being held at 20°C for 17 hours, increases in the plate counts occurred, which in Experiments 1 and 2 were particularly marked with the portions which had been cooled and refrigerated only. This may have been due in part to multiplication of normal survivors, but it seems probable that it was also the result of the growth of organisms which were "attenuated" by the treatment to such an extent that they were unable to grow until after a pre-testing period, during which time they were able to regain their normal vitality.

3. Unlike similar experiments with B. cereus, heat-treated samples of Aerobacter aerogenes, held for 24 hours at atmospheric temperature, failed to show an increase over the original plate counts. It seems therefore that all surviving organisms grew immediately after

treatment and that there were no "attenuated" organisms present.

The reduction in the plate count which followed freezing without heat-treatment, however, apparently caused many of the organisms to be in a state of "dormancy" from which they recovered only after a period of atmospheric temperature.

Sub-section 2: Methylene Blue Reduction by Aerobic Spore-forming Bacilli and other Organisms isolated from Ice Cream.

A. Methylene Blue Reduction by Aerobic Spore-forming Bacilli.

It has been suggested by previous workers that the ability of aerobic spore-forming bacilli to bring about rapid reduction of methylene blue may be responsible for unjustifiably low grading of ice creams when the methylene blue test is used for routine work. The dried-milk powder from which ice cream is often prepared, nearly always contains these organisms in varying amounts and since the heating of the ice cream mix is not sufficient to destroy the spores it is possible that however hygienic the methods of manufacture and storage may be, the grading based on the methylene blue test will be low.

If small numbers of these organisms are capable of bringing about rapid reduction of the dye then the criticism that the sample may be unjustifiably condemned is a sound one. If on the other hand, large numbers of the organisms are necessary to reduce the dye then a resulting low grade is justified since, whatever the cause large numbers of these organisms should not be present in a wholesome food. Topley & Wilson(1946) suggest that certain food-poisoning outbreaks which have

occurred from time to time and from which no pathogenic organisms were isolated may have resulted from toxic substances produced by large numbers of aerobic spore-bearing organisms present in the food. In the report on ice cream testing of the Public Health Laboratory Service (1948) it is stated that toxins produced by aerobic spore-bearing organisms are capable of producing diarrhoea and sickness.

In order to determine to what extent these organisms may affect the methylene blue reduction time, the following investigation was carried out :-

EXPERIMENTAL.

To determine the ability of Bacillus cereus to bring about reduction of methylene blue.

1. Method. The organisms were grown on an agar slope for 24 hours and the resulting growth washed off with 10 ml. ¼ strength Ringer solution. The suspension was centrifuged, the supernatant solution poured off and the deposit resuspended in 10 ml. ¼ strength Ringer solution.

Doubling-dilutions were then prepared from 1 in 2 to 1 in 2,048, using sterile ¼ strength Ringer solution as a diluent.

The Methylene Blue Test:

A series of 12 test tubes were set up, each containing 8 ml. sterile peptone water and 1 ml. methylene blue solution. To the first tube was added 1 ml. undiluted suspension of B. cereus, and to each of the other eleven was added 1 ml. of one of the prepared dilutions of the suspension.

In order to determine the number of organisms present in the original suspension, plate counts were carried out on ten-fold dilutions up to 1 in 10,000,000. The resulting figure was approximately 33,000,000 organisms per ml. From it were determined the approximate number of organisms added with each ml. of diluted suspension. The tubes were placed in a water bath at 20°C at 5 p.m. as in the recommended test and were examined after 5 hours at this temperature. After 17 hours they were transferred to the 37°C water bath and examined every half hour, until reduction was complete. In all tubes reduction was considered complete when the blue colour had disappeared from the whole column of liquid or up to within 5 m.m. of the surface.

Results:

From Table XXV. it is seen that the most rapid reduction took place in tubes 1, 2 and 3 when the colour had almost completely disappeared after five hours at

TABLE XXV.

Methylene-blue Reduction by B. cereus in peptone water.

		Methylene Blue Reduction at temperature of	time (hours)	Grade
1.	1 ml. Methylene-blue + 8 ml. Peptone-Water + 1 ml. Suspension undiluted (i.e. approximately 33,000,000 orgs. added)	20°	5	4
2.	" " " " " diluted 1:2 (" 16,500,000 " ")	"	5	4
3.	" " " " " 1:4 (" 8,250,000 " ")	"	5	4
4.	" " " " " 1:8 (" 4,125,000 " ")	"	17	4
5.	" " " " " 1:16 (" 2,062,500 " ")	"	17	4
6.	" " " " " 1:32 (" 1,031,250 " ")	"	17	4
7.	" " " " " 1:64 (" 515,625 " ")	37°	1	3
8.	" " " " " 1:128 (" 257,812 " ")	"	2	3
9.	" " " " " 1:256 (" 128,906 " ")	"	2	3
10.	" " " " " 1:512 (" 64,453 " ")	"	2	3
11.	" " " " " 1:1024 (" 32,226 " ")	"	3	2
12.	" " " " " 1:2048 (" 16,113 " ")	"	3	2

24 hour agar slope culture suspended in 10 ml. ¼ strength Ringer solution.

20°C. In these three tubes the total number of organisms added were approximately 33,000,000, 16,500,000 and 8,250,000. Tubes 4, 5 and 6 were reduced at the time of their removal from the 20°C water-bath after seventeen hours. They had originally contained approximately 4,000,000, 2,000,000 and 1,000,000 organisms. Tube 7, which had had 500,000 organisms added,showed reduction after one hour at 37°C and Tubes 8, 9 and 10 after two hours at 37°C. The numbers of organisms added to these three tubes were approximately 257,000, 129,000 and 64,000 respectively. Tubes 11 and 12 each containing originally approximately 32,000 and 16,000 organisms showed reduction after three hours at 37°C. These results as applied to ice cream would give the following grades:- Nos. 1-6 Grade 4, Nos. 7-10 Grade 3 and Nos. 11-12 Grade 2.

In judging the quality of ice cream, Grades 3 and 4 are considered to be unsatisfactory according to the English recommendations, whereas in Scotland a plate count of 100,000 organisms per ml. is the suggested maximum allowable. Since in the recommended method 2 ml. of ice cream are added to each methylene

blue reduction tube, it is reasonable to suppose that if the two tests are comparable, a Grade 3 or 4 result would arise from approximately 200,000 organisms originally present in the 2 ml. of ice cream. It is known that certain organisms are more rapid reducers than others and if it is found that sporing organisms fall into this category their presence in small numbers in ice cream samples might bring about false results.

From the results in Table XXV it is observed that in order to produce a Grade 3 result: a minimum of 64,000 organisms had to be added and for a Grade 4 result the minimum number of organisms added was approximately 1,000,000.

The figure 33,000,000 for the number of organisms originally present in the suspension was calculated from the plate count of a 1:100,000 dilution: the next highest dilution viz. 1:10,000 gave plate counts which were too high to be counted so that 33,000,000 is probably less than was actually the case, certainly not more, and consequently the figure of 64,000 organisms in tube 10 may be somewhat less than the true one. Nevertheless, even this figure suggests that the reducing capacity of B. cereus is at least as high as that of a normal mixed bacteriological flora in ice

cream on which the grading is based.

The test was repeated, this time with the addition of 2 ml. of ice cream. Owing to the fact that sterilisation of ice cream is difficult to achieve without bringing about caramelisation of the product, this was not done. The sample however was tested by the plate count and coliform count as well as by the methylene blue test and without the addition of B. cereus so that the effect of the addition of the organism could be judged. The plate count showed that approximately 6,000 organisms per ml. were present, no coliform organisms were detected in the first instance, but when tested a second time after holding at 20°C for 12 hours they were found to be present in two of the three tubes. No increase was observed in the total count at the time of the second test. The methylene blue was reduced by the organisms in the ice cream in 4¾ hours, resulting in a grading of 1 (a border-line Grade 1).

Table XXVI shows that the addition of approximately 3,000 B. cereus resulted in a methylene blue reduction time of less than 4 hours and a corresponding grading of 2. Grade 2 results were also obtained after the addition of approximately 6,000, 12,000,

TABLE XXVI.

Methylene-blue Reduction by B. cereus in Ice Cream.

No.								Methylene Blue Reduction at Temperature of	time (hours)	Grade
1.	1 ml. Methylene-blue	6 ml. Ringer's solution	2 ml. ice cream	1 ml. B. cereus suspension			(i.e. approximately 6,400,000 orgs. added)	20°	16	4
2.	"	"	"	"	diluted 1:2		(" 3,200,000 " ")	"	17	4
3.	"	"	"	"	"	1:4	(" 1,600,000 " ")	"	17	4
4.	"	"	"	"	"	1:8	(" 800,000 " ")	37°	¼	4
5.	"	"	"	"	"	1:16	(" 400,000 " ")	"	1	3
6.	"	"	"	"	"	1:32	(" 200,000 " ")	"	1½	3
7.	"	"	"	"	"	1:64	(" 100,000 " ")	"	2	3
8.	"	"	"	"	"	1:128	(" 50,000 " ")	"	2½ - 4	2
9.	"	"	"	"	"	1:256	(" 25,000 " ")	"	"	2
10.	"	"	"	"	"	1:512	(" 12,500 " ")	"	"	2
11.	"	"	"	"	"	1:1024	(" 6,250 " ")	"	"	2
12	"	"	"	"	"	1:2048	(" 3,125 " " ")	"	"	2
13.	"	7 ml.	"					"	4¾	1

24 hour agar slope culture suspended in 10 ml. ¼ strength Ringer Solution.

25,000 and 50,000 B. cereus. These figures are comparable with those obtained in the previous experiment where approximately 16,000 and 32,000 organisms added to peptone water and methylene blue resulted in reduction of the dye within 3 hours and a grading of 2 in each case.

In the present experiment a Grade 3 result was obtained after the addition of 100,000, 200,000 and 400,000 organisms respectively which is comparable with the grading based on the results of the previous experiment, where approximately 64,000, 129,000, 257,000, and 500,000 organisms brought about reduction in from 1 to 2 hours. The addition of 800,000 organisms was necessary to bring about reduction within ¼ hour and a resulting grading of 4 which is comparable with the figure of 1,000,000 in the previous experiment necessary to produce reduction during the 17 hours incubation at 20°C.

These two experiments appear to indicate that although as few as 16,000 B. cereus added to sterile media and 3,000 B. cereus added to a Grade 1 (borderline) ice cream may bring about reduction in 2½ - 4 hours giving a Grade 2 result, as many as 50,000 may be added without lowering the grade further. At least

65,000 to 100,000 organisms are necessary to lower the grade to 3 and 800,000 to 1,000,000 to give a Grade 4 result.

In view of these findings it seems that B. cereus in ice cream is not liable to give an unjustifiably low grading of ice cream samples, since fairly large numbers must be present in order to bring about rapid reduction of methylene blue.

B. Methylene-Blue Reduction by Organisms Isolated from Ice Cream.

EXPERIMENTAL:

In order to test and compare the abilities to reduce methylene-blue, of various bacteria isolated from ice cream, the following experiments were set up:-

1. Method:- A sample of high quality ice cream was allowed to melt at room temperature and 10 ml. quantities were then pipetted into each of 7 test tubes (Nos. 1-7). To each of the tubes 2 to 7 were added 1 ml. of an agar slope culture of one of the organisms previously isolated from ice cream, emulsified in sterile tap water to give an opacity equivalent to Brown's opacity tube No. 3.

The organisms used were those isolated from ice cream No. 253 and included B. cereus, Achromobacter delicatum, Flavobacterium diffusion and Streptococcus faecalis.

Plate counts of the contents of each tube were made immediately after the addition of the organisms, and again after the tubes had been held in a water-bath at 20°C for 17 hours. The methylene-blue reduction test was set up in the manner recommended by the Ministry of Health, 2 ml. of the inoculated ice cream being

TABLE XXVII.

Methylene blue Reduction by Various Organisms Isolated from Ice Cream No. 53.

Tube	Plate Count (thousands) Tested		Meth. Blue Reduction	
	Immed.	After 17 hrs.	Time (hrs.)	Grade
1. Ice Cream	3.8	68	7½	1
2. " " + 53(1) B. cereus	500	500	0	4
3. " " + 53(2) Achromobacter delicatum	3.9	31	7½	1
4. " " + 53(3) B. cereus	500	500	2	3
5. " " + 53(4) Strep. faecalis	500	500	0	4
6. " " + 53(6) Flavobacterium diffusum	500	500	7½	1

The Plate Count test was carried out (a) immediately after the addition of the cultures to the melted samples and (b) after the inoculated sample had been held at 20°C for 17 hours.

added to tubes containing 7 ml. ¼ strength Ringer solution and 1 ml. methylene-blue solution. After being held at 20°C for 17 hours, the tubes were placed in a water-bath at 37°C and examined and inverted every half-hour until reduction was complete.

The results of the tests are shown in Table XXVII where they may be compared with the results of similar tests on the ice cream in Tube 1 to which no organisms were added.

In every case with the exception of No. 3 the added organisms established themselves in the ice cream and the resulting plate counts were very high. Since similar numbers of organisms were added to the tubes of ice cream, the differences in the times required to bring about reduction of methylene blue must be due mainly to the nature of the organisms rather than to their numbers.

The uninoculated ice cream with a plate count of 3,800 organisms per ml. failed to reduce the dye in 7½ hours and was accordingly Grade 1. The addition of Flavobacterium diffusum in numbers greater than 500,000 per ml. had no appreciable effect on the reduction time. Two cultures of B. cereus brought about reduction of the dye in (a) 2 hours and (b) during the pre-incubation

period at 20°C thus lowering the grade of the ice cream in each case to Grade 3 and Grade 4 respectively. Streptococcus faecalis also, was found to be a rapid reducer of methylene blue and its addition to the sample produced complete reduction during the pre-incubation period and consequently lowered the grade of the sample to 4.

2. A similar experiment was carried out with organisms isolated from ice cream No. 54. The method was the same except that the dilutions prepared for the plate count test were increased to 1/10,000 and 1/100,000 so that a more accurate count could be obtained. The results are shown in Table XXVIII.

Three of the strains isolated from this sample of ice cream were identical in character and were identified as Paracolobacterum aerogenoides while two others proved to be Alcaligenes metalcaligenes. All strains were tested in order to confirm the consistency of the results.

The ice cream to which the cultures were added gave a low plate count of 900 organisms per ml. and a methylene blue reduction time of more than 9 hours. The addition of large numbers of Paracolobactrum failed to

TABLE XXVIII.

Methylene blue Reduction by Various Organisms Isolated from Ice Cream No. 54.

Tube	Plate Count (thousands/ml.) Tested		Meth. Blue Reduction	
	Immed.	After 17 hrs.	Time (hrs.)	Grade
1. Ice cream	0.9	5.7	9	1
2. " " + 54(1) Paracolobactrum aerogenoides	180,000	320,000	9	1
3. " " + 54(2) Alcaligenes metalcaligenes	37,000	35,000	0 - ½	4
4. " " + 54(3) Alcaligenes metalcaligenes	60,000	76,000	0 - ½	4
5. " " + 54(4) Paracolobactrum aerogenoides	145,000	420,000	9	1
6. " " + 54(5) Paracolobactrum aerogenoides	70,000	364,000	9	1
also 7. " " + 53(2) Achromobacter delicatum *	less than 10	less than 10	8	1

*(repeated from previous experiment).

lower the reduction time to 9 hours or less in any of the three cases examined and all remained accordingly, Grade I. The two strains of Alcaligenes, on the other hand lowered the time of reduction to less than ½ hour and the grade to 4 in each case.

The strain of Achromobacter delicatum No. 53 (2) which appeared to have failed to establish itself in the ice cream in the previous experiment was used to inoculate a tube of the ice cream in this experiment. The resulting plate count was less than 10,000 both when the sample was tested immediately after inoculation and also after 17 hours at 20°C, but the time required to reduce methylene blue was slightly less than for the uninoculated ice cream, reduction being complete in 8 hours. It is possible that this organisms, although not capable of growing well on agar medium at 37°C may bring about slow reduction of methylene blue as a result of its metabolism during the pre-testing period at 20°C.

3. The experiment was repeated with a fresh sample of ice cream inoculated with cultures:- 55 (1), 55 (6) and 55 (7) isolated from a sample of ice cream No. 55. The results are shown in Table XXIX.

It is seen that Achromobacter liquefaciens

TABLE XXIX.

Methylene blue Reduction by Various Organisms Isolated from Ice Cream No. 55.

Tube	Plate Count (thousands/ml.)		Meth. blue Reduction	
	Immed.	After 17 hrs.	Time (hrs.)	Grade
1. Ice cream	2.4	24	7	1
2. " " + 55 (1) Achromobacter liquefaciens	20,000	200,000	7	1
3. " " + 55 (6) Aerobacter cloacae	400,000	800,000	0	4
4. " " + 55 (7) Alcaligenes bookeri	400,000	640,000	0	4

although present in fairly high numbers failed to produce any significant change in the methylene blue reduction time.

A species of Alcaligenes on the other hand and Aerobacter cloacae reduced methylene blue by the time of completion of the pre-testing period at 20°C and accordingly lowered the grade of the sample to Grade 4. 4. Table XXX shows the results of the same experiment using a fresh sample of ice cream and cultures of organisms isolated from ice cream samples Nos. 55 and 56. No plate counts were made in this instance except on the uninoculated ice cream sample, the result of which was 4,000 per ml.; the methylene blue reduction time was 6½ hours and the resulting grade, I.

The addition of Achromobacter liquefaciens No. 55 (4) and Paracolobactrum aerogenoides No. 56 (4), failed to lower the reduction time. A species of Sarcina No. 56 (2) although producing partial reduction in 4 hours completed the reduction only after 6½ hours so that there was no change in the resulting grading of the sample. Aerobacter cloacae lowered the reduction time to 2 hours, and the grading to 3, while a species of Corynebacterium No. 56 (3) brought about complete reduction within the 17 hours at 20°C and consequently reduced the grading to 4.

TABLE XXX.

Methylene blue Reduction by Various Organisms Isolated from Ice Cream Nos. 55 & 56.

Tube	Methylene blue Reduction	
	Time (hrs.)	Grade
1. Ice cream (4,000 organisms per ml.)	6½	1
2. " " + 55 (4) Achromobacter liquefaciens	6½	1
3. " " + 56 (2) Sarcina sp.	4 - 6½	1
4. " " + 56 (3) Corynebacterium sp.	0	4
5. " " + 56 (4) Paracolobactrum aerogenoides	8	1
6. " " + 56 (5) Aerobacter cloacae	2	3

5. Table XXXI shows the results of the same experiment with two strains of Achromobacter Nos. 55 (2) and 55 (4) and a strain of Aerobacter cloacae, No. 55 (8).

No change was found to occur in the times required to bring about reduction of methylene blue and there was no rise in the plate count of the samples, even after the addition of large numbers of bacilli from cultures of the two strains of Achromobacter liquefaciens. This confirmed the results obtained previously in Experiment 4 for Achromobacter liquefaciens No. 55 (4). The addition to the sample, of Aerobacter cloacae, on the other hand brought about reduction of methylene blue in less than ½ hour with the result that the grading of the sample was lowered from Grade I to Grade 4.

Summary:

1. The modified methylene blue reduction test was carried out on samples of ice cream of a high bacteriological quality, and on portions of the same samples to which were added suspensions, of comparable opacity, of different organisms isolated from ice cream.

2. The addition to the samples of some species brought about a decrease in the methylene blue reduction times,

TABLE XXXI.

Methylene blue Reduction by Various Organisms Isolated from Ice Cream No. 55.

Tube	Plate Count (thousands/ml.)	Meth.-blue Reduction	
	Immed.	Time (hrs.)	Grade
1. Ice cream	2.8	7	1
2. " " + 55(2) Achromobacter liquefaciens	10	7	1
3. " " + 55(4) Achromobacter liquefaciens	10	7	1
4. " " + 55(8) Aerobacter cloacae	230,000	½	4

whereas other species failed to have any apparent effect on the reduction of the dye.

3. Since similarly large numbers of organisms were added to the sample in each experiment, any differences in the reduction times must have been due mainly to the nature of the organisms rather than to small differences in their numbers.

4. No change in the methylene blue reduction time, and therefore no lowering of the grade of the samples resulted from the addition of cultures of the following organisms to ice cream:-

Achromobacter delicatum

Achromobacter liquefaciens

Flavobacterium diffusum

Sarcina sp.

Paracolobactrum aerogenoides.

5. The time required to bring about complete reduction of methylene blue was considerably lessened, and the resulting grades of the samples lowered to 3 or 4, by the addition of the following organisms:

Alcaligenes metalcaligenes

Alcaligenes bookeri

Streptococcus faecalis

Aerobacter cloacae

Bacillus cereus

Corynebacterium sp.

SECTION IV.

GENERAL DISCUSSION AND RECOMMENDATIONS.

SECTION IV.

GENERAL DISCUSSION AND RECOMMENDATIONS.

Two criticisms have been levelled against the use of the modified form of the methylene blue reduction test, recommended by the Ministry of Health for the routine examination of ice cream samples. They are as follows:-

(a) The results of the methylene blue test do not always correlate with the bacterial counts of corresponding samples, as judged by the plate count test, and samples graded 3 and 4 by the methylene blue test have sometimes been found to give very low plate counts.

(b) Aerobic spore-forming bacilli, originating from the ingredients of the ice cream "mix" may resist pasteurisation and bring about rapid reduction of methylene blue, even when present in small numbers, thereby giving a false impression of the hygienic quality of the ice cream.

In dealing first of all with criticism "a", the present investigation has shown that if the samples are retained for a period at a controlled temperature of 20°C, before carrying out the plate count test, closer correlation between the results of the two tests is

brought about. It may be argued that by so doing, the samples are not being tested in the same condition as they are in when received by the consumer, but on the other hand, the plate count test carried out immediately after the sample has been in a frozen condition, may not give a true indication of the quality of the ice cream at that time. Although it is recognised that the plate count of a sample is not identical with its total bacterial content, but merely represents the number of organisms present, which are capable of growing under the conditions of the test, yet, if it is to have any value it must be indicative of the total, and should be capable of separating out samples which have received different degrees of contamination.

The danger of relying too much on plate counts for judging ice cream is that a low count may not necessarily mean that the ice cream is free from contamination. It appears that ice cream may contain the following types of organisms:- (a) spore-forming bacilli in a dormant state as a result of the heat-treatment; (b) vegetative organisms which have survived heat-treatment but are "attenuated"* as a result of it, and (c) organisms which have contaminated the ice cream subsequent to

* For a definition of the term "attenuated", as used throughout this work, refer to page 205.

heat-treatment and are "attenuated" as a result of cooling and freezing. Since"attenuation" may result in a slowing down of its growth rate, none of these organisms may grow when transferred immediately to an agar medium,but by holding for a time at approximately atmospheric temperature before testing, the organisms may regain their normal function and be revealed by subsequent plate count tests. Results are given in Section I. which show that if samples with similarly low plate counts, when tested immediately after melting, are tested again after periods of 6, 12, 18 and 24 hours, they can be divided into:- (a) those samples which have no significant rise in their plate counts after the pre-testing periods, and (b) those with counts which show a marked increase during the same time. It appears that all of these samples cannot be of a similarly high quality, in spite of their first satisfactory results, and the increases in the counts of some is probably explained by the growth of organisms, which failed to grow in the first instance, because of their being in an "attenuated" condition as a result of the heating and cooling processes.

To illustrate the effects that heat-treatment and

subsequent freezing may have on the bacterial content of an ice cream mix," the results of experiments involving the heating and freezing of cultures of Bacillus cereus and Aerobacter aerogenes are given in Section III., sub-section I. Freezing independent of heat-treatment is able to produce an apparent reduction in the bacterial count. By holding the treated cultures at 20°C for 24 hours and retesting, the resulting increases in the counts appear to be greater than can be accounted for by the multiplication of the normal survivors and are thought to be due to the growth of organisms which failed to grow when first tested, because of their "attenuated" or "dormant" condition.

Pre-incubation of samples at 20°C for 17 hours was introduced into the technique of the methylene blue reduction test, in order that latent contamination should be revealed. It is not surprising that there should not be complete agreement between the results of this test, and the plate counts of corresponding samples tested immediately after being in a frozen condition, i.e. 17 hours earlier. Results of the investigation described in Section I., subsection 3, show that a closer correlation is attained between the results when the plate count test is carried out after the samples

have been held at 20°C for 6 and 12 hours, although the methylene blue test tends to be slightly more exacting in judging the samples than is the plate count method at that time. After pre-testing periods of 18 and 24 hours, the plate count test becomes more exacting than the methylene blue reduction test, and if a pre-testing period as long as 18 hours were introduced as a modification, in carrying out the plate count test, too many samples would fail to reach the provisional standard of not more than 100,000 organisms per ml. Of the four pre-testing periods investigated, the 12 hour period allowed the plate count test to give closest correlation with the methylene blue reduction times and it is possible that even closer correlation would be achieved if the samples were held for a period of between 12 and 18 hours before testing. Such a procedure however is not practicable, as it presents an awkward interval of time for routine testing purposes. It is recommended, instead, that if the plate count test is to continue to be used for the examination of ice cream samples, a pre-testing period of 6 hours at 20°C should be introduced in order that latent contamination in the sample should be revealed as far as possible. This would necessitate maintaining samples in a frozen

condition until 9 or 10 a.m. on the day following sampling and then holding them for 6 hours at 20°C before testing.

By introducing a pre-testing period of 6 hours before carrying out the plate count test, close agreement between the results of the two methods of testing was found in 90% of the samples examined, the majority of the discrepancies being due to the fact that the methylene blue test was slightly more exacting in judging the samples, than the plate count test carried out at that time.

With regard to criticism "b", which condemns the methylene blue reduction test on the grounds that sporing bacilli in small numbers may bring about rapid reduction of methylene blue, and thereby give an unjustifiably low grading to the sample, work described in Section II. shows that spore-forming bacilli particularly Bacillus cereus are indeed very common in ice cream samples, no doubt derived from the ingredients of the "mix". On the other hand they are as frequently present in Grade 1 samples as in those of the lower grades, although in apparently fewer numbers, which suggests that for a low grading to result from their presence, fairly large numbers would have to be present

initially. This is confirmed in Section III. in which results are given which show that in order to produce a Grade 3 result, the minimum number of organisms of Bacillus cereus required, was approximately the same as the maximum bacterial count allowable under the Scottish provisional standards for ice cream, based on the plate count test, while for a Grade 4 result a minimum of about 1,000,000 organisms was necessary.

Previous workers who found that apparently small number of Bacillus cereus were capable of producing rapid reduction of the dye, had presumably carried out the plate count tests on samples when the organisms were still in a dormant state, and, had a pre-testing period been allowed, it is probable that subsequent testing would have revealed the presence of many more bacilli.

The presence of spore-forming bacilli in large numbers in the ice cream ingredients is undesirable, since owing to their resistant nature, the organisms may build up in the ice cream plant and form sources of contamination, which may be difficult to eradicate. Their growth in ice cream "mixes" may result in the formation of toxic substances capable of causing

outbreaks of diarrhoea and sickness.

It appears, therefore, that a Grade 3 or Grade 4 result, mainly due to the reduction of methylene blue by spore-forming bacilli, far from being unjustifiable, is of value in indicating the need in such cases for increased supervision and control of the bacteriological quality of the ingredients, and the cleanliness and sterility of the plant. Plate counts, on the other hand, when carried out immediately after the ice cream samples have been in a frozen condition, will fail to show up this undesirable form of contamination.

As regards bacteriological methods for ice cream plant control, and detection of contamination of the "mix" after heat-treatment, the results of experiments described in Section I., sub-section 5 and 6 seem to indicate that the methylene blue reduction test is particularly sensitive to even small degrees of contamination. Provided the actual times required for complete reduction of the methylene blue are taken into consideration, and samples taken at different stages of the manufacturing process are compared with one another on that basis, rather than on the resulting grades alone, information may be gained as to the location of even minor sources of contamination in the plant. The plate

count test was found to have little value for plant control work when samples were tested immediately after the ice cream had been heated or frozen. After the samples had been held for a time at 20°C however, the test became more sensitive to different degrees of contamination. It was shown too, that the presumptive test for coliform bacilli, on samples taken at different stages during ice cream manufacture may be of value in showing up sources of contamination particularly if the samples are given a "pre-testing" period at 20°C.

The test for coliform bacilli as applied to the examination of milk is of value in detecting (a) inadequate pasteurisation and (b) contamination after pasteurisation. For ice cream work, however, owing to increased resistance of the organisms to heat, resulting from the protection afforded by the high sugar content, coliform bacilli may survive the methods of pasteurisation laid down in the ice cream regulations. Their presence in the finished product, therefore, must be interpreted with caution. Samples taken at different stages, from the heating unit onwards will reveal the source of these organisms, especially if samples are held for a time at 20°C before testing is begun.

It is not easy to assess the significance of coliform bacilli in ice cream. Unlike their presence in water samples, they cannot be taken to indicate direct excretal pollution. They probably originate in most cases from contaminated ingredients, and may resist heat-treatment either because of defects in the efficiency of the process, or as shown by previous workers, because of the protective action of the sugar in the "mix". If they are absent from a sample taken directly from the heated "mix" (the sample having been held for a time at 20°C before being tested) and occur in the finished product, this presumably indicates that contamination has occurred at some stage beyond the heating unit, probably as a result of inadequate cleansing and sterilising of the plant.

Escherichia coli (faecal B. coli) sometimes occur in ice cream. As described in Section II., sub-section 3, five strains were isolated from 53 samples of ice cream which gave positive reactions in the presumptive test. They accounted for 8% of the total number of 61 different strains of coliform bacilli isolated from the samples. They must be presumed to have arisen from contamination from the workers' hands and clothing and the significance of their presence in ice

cream should not be under-rated. In view of the risks involved from contamination of ice cream by carriers of pathogenic organisms, it is of utmost importance that, as far as is possible, the product should be protected from contamination of human origin.

Of academic interest is the fact that Aerobacter cloacae was more common in ice cream than Aerobacter aerogenes and accounted for 58% of the total Aerobacter strains.

Unusual features of the strains of Aerobacter aerogenes isolated from ice cream, were their slowness in producing mucoid colonies when grown at 35°C and their failure to produce gas in inositol. Some strains of Aerobacter cloacae also showed irregularities in failing to liquefy gelatine, even after four months, in failing to produce mucoid colonies at 35°C within 24 hours, although becoming mucoid later, and in being slow to produce gas from "sugars". These abnormal features may be manifestations of changes brought about in the organisms, as a result of the heat-treatment and freezing, to which they were submitted in the ice cream, although experiments described in the appendix to Section II., involving the heating and sudden cooling and freezing of cultures of typical Aerobacter aerogenes, isolated from sources other than ice cream, failed to produce non-mucoid varients.

SUMMARY and CONCLUSIONS:

1. A study of various bacteriological methods for grading ice cream, indicated that by holding the samples for a time at a controlled temperature of 20°C, before carrying out the plate count test, the results correlate more closely with those of the modified methylene blue reduction test, recommended by the Ministry of Health for ice cream testing.

2. Of four "pre-testing" periods of 6, 12, 18 and 24 hours respectively, the closest correlation between the results occurred, when samples were held for 12 hours at 20°C before the plate count tests were carried out. Owing to the practical difficulties involved in holding samples for 12 hours, before testing, a period of 6 hours is suggested instead, for routine work. During the investigation, 90% of the results were rendered comparable by this means, the discrepancies being due mainly to the fact that the dye reduction test was slightly more exacting in judging the samples, than the plate count test at that time.

3. The correlation between the results of the presumptive test for coliform bacilli and the methylene blue reduction times was similarly greater, when a "pre-testing" period was allowed.

4. The holding of ice cream samples at 20°C for a period before testing is recommended, to ensure that contaminating organisms, which have been rendered "dormant" or "attenuated" by heat treatment and subsequent freezing, may regain their normal viltality and so be revealed by the tests.

5. Experiments are described which indicate that organisms appear to be "attenuated" by heat-treatment and/or sudden cooling and freezing.

6. Comparisons of plate counts of ice cream samples tested immediately after melting, and again after being held for periods at 20°C before the test is carried out, show how these"pre-testing" periodsrender the test more sensitive in differentiating samples with different degrees of contamination.

7. For the bacteriological control of ice cream plant, the plate count test carried out immediately after the samples were received in the laboratory, was shown to be incapable of detecting sources of contamination. The test became more sensitive when a pre-testing period was introduced. The methylene blue reduction test proved to be sufficiently sensitive to

show up contamination, especially if the actual times required to bring about complete reduction of the dye were noted, and used to compare samples taken at different stages of the manufacture.

8. Within the limits of the test, the time required to bring about complete reduction of methylene blue depends on the numbers rather than on the nature of the organisms in the ice cream. Although certain organisms, including Bacillus cereus, are able to reduce the dye more rapidly than others, experiments showed that the number of organisms required to lower the samples to Grades 3 or 4 is greater than should be permissible in ice cream.

9. Systematic examinations of samples of ice cream revealed a variety of organisms occurring in all grades, but organisms from samples of Grades 1 and 2 were rarely isolated until after the time required to bring about complete reduction of methylene blue, and were presumably present initially in very small numbers.

10. Coliform organisms isolated from ice cream included 8% Escherichia coli. Aerobacter cloacae was more frequently present than Aerobacter aerogenes and accounted for 34% of the total number of coliform strains isolated.

Results of biochemical tests showed irregularities in certain features of many of the *aerogenes* and some of the *cloacae* species isolated. These included slowness in producing mucoid colonies by both species, inability of *Aerobacter aerogenes* to ferment inositol, and the failure of some *cloacae* strains to liquefy gelatine.

It is tentatively suggested that these variations in the nature of the organisms, may have been induced by the action of heat-treatment and freezing to which they were subjected in the ice cream.

BIBLIOGRAPHY.

Allen, P.W. (1923) J. Bact., 8, 555.

Anderson, E.B. (1935) J. Roy. San. Inst., 55, 592.

Anzulovic, J.V. (1932) J. Bact., 23, 56.

Bancroft, W.D. and Richter, G.H. (1931) J. Phys. Chem., 35, 511.

Bardsley, D.A. (1934) J. Hyg., Camb., 34, 38.

Bardsley, D.A. (1938) ibid., 38, 527.

Beavens, E.A. (1930) J. Dairy Sci., 13, 91.

Breed, R.S. (1911) Centralbl. f. Bakt., Abt. II., 30, 337.

Buchan, G.F. (1910) J. Hyg., 10, 93.

Circular (1947) "Ice Cream (Heat Treatment, etc.) Regulations, 1947". Circular 69/47, Min. Hlth., London.

Circular (1948) "Ice Cream (Scotland) Regulations 1948", Circular No. 43/1948. Dept. Hlth., Scotland.

Clayson, D.H.F. and Pirie, D.G. (1948) J. Soc. Chem. Ind., 67, 147.

Cooper, K.E. (1949) Munic. J. London, 57, 905.

Cranfield, J. (1948) J. Dairy Ind., 13, vii, 698.
ibid., 13, viii, 800.

Duguid, J.P. (1951) J. Path., Bact., 63, 673.

Eijkman, C. (1908) Biochem. Z., 11, 12.

Evans, D.I. (1947) Med. Offr., 77, 39.

Fabian, F.W. (1925) J. Dairy Sci., 8, 246.

Fabian, F.W. and Coulter, E.W. (1930) J. Dairy Sci., 13, 273.

Fay, A.C. (1926) J. Bact., 11, 96.

Fay, A.C. (1930) J. Dairy Sci., 13, 40.

Fay, A.C. (1933) ibid., 16, 311.

Fay, A.C. (1934) J. Agr. Res., 48, 453.

Foltz, V.D. and Martin, W.H. (1938) J. Dairy Sci., 21, 289.

Fournelle, H.J. and Macy, H. (1942) J. Dairy Sci., 25, 475.

Fuchs, A.W. (1941) J. Milk. Tech. 4, 26.

Griffin, A.M. and Stuart, C.A. (1940) J. Bact., 40, 83.

Hoather, R.C. and Bullough, W.A. (1951) Brit. Med. J. May 12, 1084.

Kliger, I.J. (1914) J. Inf. Dis., 15, 187.

Laws, A.J. (1948) Med. Off., 79, 89.

Malcolm, J.F. (1935) J. Dairy Res. 6, 383.

Malcolm, J.F. (1938) J. Hyg. 38, 395.

Memorandum (1948) "Ice Cream, Memorandum on the Hygienic Control of Premises and Equipment". Memo. No. 43 (Tech.) Dept. Hlth. Scotland.

Neild-Cook, J. (1896) Public Health, 7, 252.

Nelson, F.E. (1943) J. Bact., 45, 395.

Nelson, F.E. (1944) J. Dairy Sc., 27, 993.

Pederson, C.S. and Breed, R.S. (1940) Am.J. Pub. Hlth., 30, 162.

Pederson, C.S. and Yale, M.W. (1934) ibid., 24, 477.

Prescott, S.C. and Breed, R.S. (1910) J. Inf. Dis., 7, 632.

Regulations (1940) "Frozen Desserts Ordinance and Code". U.S. Public Health Service,
(1940) J. Milk Tech., 3, 44.
(1941) ibid., 4, 26.

Regulations (1947) "Ice Cream (Heat Treatment etc.) Regulations, 1947". Statutory Rules and Orders, No. 612.

Regulations (1948) "The Ice Cream (Scotland) Regulations, 1948". Statutory Instruments 1948, No. 960 (S. 68).

Report (1879) "Sanitary Conditions of the Italian Quarter". Lancet, 2, 590.

Report (1894) "The Bacteriology of Ice Cream". Lancet, 2, 862.

Report (1937) "Ice Cream: Regulations and Standards, Manufacturing Methods". Am. J. Pub. Hlth. (1937) 27. Year Book (1936-1937), 53.

Report (1947) "The Bacteriological Examination and Grading of Ice Cream". Mon. Bull. Min. Hlth., London, 6, 60.
(1948) ibid., 7, 84.
(1949) ibid., 8, 155.
(1950) ibid., 9, 231.

Savage, W. (1938) J. Hyg. 38, 331.

Williams, G.C., Swift S., Vollim, R.L., and Wilson, G.S. (1946) Mon. Bull. Min. Hlth., London, 5, 17.

White, A.H. (1938) Sci. Agr. 19, 69.

Wilkinson, J. (1899) Pub. Hlth., 11, 255.

Wilson, G.S., Twigg, R.S., Wright, R.C., Hendry, C.B., Cowell, M.P., and Maier, I. (1935) Spec. Rep. Ser. Med. Res. Coun., London, No. 206.

Wilson, G.S. (1948) Med. Off., 79, 261.

Yale, M.W. and Pederson, G.S. (1936) Am. J. Pub. Hlth., 26, 344.

Reference Books.

American Public Health Association. "Standard Methods for the Examination of Dairy Products". Seventh and Ninth Editiens (1934 and 1938) A.P.H.A. New York.

Bergey. "Manual of Determinative Bacteriology". Sixth Edition (1948). Baillière, Tindall and Cox, London.

Hammer, B.W. "Dairy Bacteriology". Third Edition (1940) John Wiley & Sons, New York.

Tanner, F.W. "Microbiology of Foods". Second Edition (1944). Gerrard Press, U.S.A.

Topley, W.W.C., and Wilson, G.S. "The Principles of Bacteriology and Immunity". Third Edition (1946). Arnold & Co., London.
